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## INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

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## EDITOR'S VIEWPOINT

Orland, Blayney, Harrison, Reyniers, Trexler, Wagner, Gordon, and Luckey (p. 147) report caries' studies in twenty-two rats raised under germ-free conditions and thirty conventionally raised rats fed the same cariogenic diet. None of the germ-free animals developed caries; twenty-eight of the conventionally raised rats developed cavities. It therefore seems, as stated by the authors, that rat dental caries is not possible in the absence of microorganisms. This difficult and tedious study is most convincing evidence that bacteria play an essential role in dental caries and that reduction in their activity should reduce decay.

Kamrin (p. 175) presents a most interesting technic for study of experimental caries. Animals may be made "parabiotic" by surgical union developing an interchange of blood supply. Preliminary studies indicate a striking similarity of caries' patterns in parabiosed pairs. A large series of studies on local and systemic factors suggest themselves for study in such animals.

Lefkowitz, Mardfin, and Bodecker present a series of three papers (p. 181, p. 189, p. 201) on the tissue culture technic applied to teeth. Although there have been previous reports on in vitro cultivation of tooth germs by various authors, the results have not been uniformly successful. The present authors use the Carrel flask as the repository for the tooth germs, and the method is designed to require a minimal amount of equipment and laboratory space. The authors have successfully cultivated rat molar tooth germs by this technic. Not only have the tooth germs survived, but two days of development have been observed. There is a vast difference between maintaining tissues in vitro situations and the continued growth and development of those tissues. The tooth germs are preferably dissected out at 19 days of embryonic growth, transferred to Carrel flasks, and supplied with chick embyo extract as a nutrient media. The development of tooth germs under these conditions can be followed very closely, and the method also may be applicable to studies of the effects of growth hormones and various other factors on the tooth germs.

Because the acid decalcification in dental caries may occur in an environment rich in glucose, Forscher and Fosdick (p. 206) studied the effect of sugar concentration on the diffusion rate of hydrogen ion through a membrane. Under conditions of their experiment, the diffusion rate of acid was inversely proportional to the concentration of sugar in the acid. It is suggested that physiocochemical factors such as this may modify the in vivo condition of dental caries.

Fosdick and Forscher (p. 212), expanding their previous experiments, demonstrated that the presence of sugar decreases the rate of diffusion of calcium and phosphate ions through a permeable membrane. The significance of this, if any, in the carious process remains to be demonstrated.

Sullivan (p. 218) presents studies of carious lesions of enamel stained by methyl green or alizarin red S. He followed the stain down the interrod areas and concluded that the interrod organic enamel may afford pathways for caries. Of course, as Sullivan suggests, the process of decalcification may precede the action on organic material, freeing the organic-inorganic bond. Studies of a chemical nature and by electronic microscopy tend to support this explanation. The variation in lesions viewed by sectioning at different planes and the technic of incident light examination add to the interest of the paper.

Sullivan (p. 231) subjected normal enamel to buffer solutions and normal and carious enamel to histochemical treatment. He concluded that the protein structure of enamel is not broken down by acid decalcification and that enamel protein contains hexosamine, suggesting a mucopolysaccharide. The route of silver nitrate and lactic acid penetration was similar. He was unable to find support for the theory that caries of the enamel is preceded by destruction of proteins.

Muhler (p. 245), after feeding cariogenic diets to two different strains of rats beginning at different ages, concluded that newly erupted teeth are particularly susceptible to a cariogenic diet. He proposes that some apparent strain differences in rats may not be genetic but rather due to diets fed during the first 4 weeks. Stannous fluoride was more effective than sodium fluoride in reducing decay during the second generation but the degree of protection was much less than in the first generation fed fluorides. The work of Elliott and Pigman (J. D. Res. 33: 27) also suggests that early use of cariogenic diets may increase caries' activity in experimental animals. This important time factor should not be neglected by investigators, and those purchasing stock rats should make themselves aware of the postweaning diet.

Attempts to produce cancer experimentally in the oral mucosa of animals have been unsuccessful in general. Salley (p. 253), in the paper which won him the Novice Award of the International Association for Dental Research at the 1953 meeting, reports successful production of carcinoma in the pouches of hamsters after painting with carcinogens. Histologically, the pouch epithelium closely resembles the oral epithelium. This study demonstrates a useful tool and method for investigations into the important field of oral carcinogenesis.

Weinmann and Macapanpan (p. 263) placed rubber dam between the first and second molars of rats for one to seventy-two hours and then studied sections of the jaws. They found that gingivitis was followed by periodontitis in the region beneath the rubber dam but not in the periodontal membranes subjected to pressure alone. They conclude that traumatism (that is, the type associated with occlusal disturbance) is not a cause of periodontitis, per se. In spite of this conclusion by the authors, we believe they demonstrated that trauma (by the rubber dam acting on the gingiva and periodontal fibers) was a cause of periodontitis in their animals. In the human being, it would be difficult to demonstrate that pressure or pull on the periodontal fibers is a sole factor in periodontitis, for gingivitis is almost universal and, except in healthy youngsters, local stresses have reduced tissue resistance.

Turesky and Glickman (p. 273) give the results of histochemical studies of the gingiva before gingivectomy and during healing in normal and vitamin C-deficient rats. Healing was disturbed by adverse influence on the basement membrane, ground substance, and collagen fibers in vitamin C deficiency. These observations demonstrate the importance of vitamin C deficiency in healing but should not be misinterpreted to mean the vitamin C therapy will *increase* gingival wound healing in the patient who is *not* vitamin C deficient.

Henry and Peyton (p. 281) present a study of the relationship between design and cutting efficiency of dental burs. Attempts have been made to eliminate trauma to odontoblastic processes (and the pulp) through changes in technic bland bases, less irritating filling materials, and other means. At the same time, dentists have left the design of burs, and their standardization, to the manufacturers. This paper demonstrates that the problem is a dental problem and deserving of thorough study.

H. B. G. R.

# USE OF THE GERMFREE ANIMAL TECHNIC IN THE STUDY OF EXPERIMENTAL DENTAL CARIES

## I. Basic Observations on Rats Reared Free of All Microorganisms

FRANK J. ORLAND, J. ROY BLAYNEY, AND R. WENDELL HARRISON

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### INTRODUCTION

DESPITE widespread interest in dental caries, and the consequent accumulation of numerous well-planned experimental investigations in search of the causes of this disease, a clear understanding of its etiology and pathogenesis is definitely lacking. There is evidence, however, of many factors both systemic and local, modifying or contributory to the carious process. Study of the interrelationships of these factors has given origin to a few attractive theories, but the validity of current theories still depends on the discovery of final proof of etiology.

The bacterial flora of the mouth, and more specifically of the carious lesion, is so complex that any potential cariogenic effects of microorganisms are constantly subject to modifying influences arising from the simultaneous action of some or all of the numerous species and varieties commonly present in this localized area. These modifying effects could be either additive or symbiotic, they could be neutralizing, or they could be either inhibitory or antibiotic. Even if the cause of dental caries is to be found in phenomena other than those associated with the metabolic activities of the local bacterial flora, as has been claimed by a few investigators, the objective demonstration of such phenomena is made exceedingly difficult, if not impossible, by the ubiquitous nature of oral microorganisms, some of which have the capacity, in vitro, to bring about a dissolution of tooth substance closely resembling in some respects certain stages in the dental caries process.

In view of the foregoing considerations, we have undertaken a series of investigations with animals which are initially free of all bacteria, but to which single or multiple strains of known bacteria can be introduced. Only by this means does it seem possible to demonstrate clearly the bacterial effect on the teeth of animals fed a diet which in normal animals induces abundant tooth decay.

This project was aided by contracts between the Office of Naval Research, Department of the Navy, and the Universities of Chicago (NR: N6 ori-20) and Notre Dame (NR: 131-167).

Received for publication, March 11, 1953.

From time to time, efforts have been made to define more or less precisely the meaning of the term "dental caries." Various definitions have been written, almost always mentioning potential causative agents. The definition proposed at a 1947 conference on dental caries,[1] although adopted unanimously by the rather formidable number in attendance, has not found universal acceptance among all investigators of this dental disease. It has seemed important at this stage of caries research, and particularly in connection with the studies here reported, to formulate a simple, yet basic concept of the dental caries process acceptable to all students of the problem, regardless of their various and sometimes diametrically opposite views of the etiology of caries. Hence, the following definition has been constructed in graded form with the individual consultation of a considerable number of research workers who hold in common at least this descriptive concept of the disease. Furthermore, since the investigations to be reported from these laboratories have a direct bearing on specific causative agents of dental caries, this definition contains no reference whatsoever to etiological factors.

### AN EXTENDED DESCRIPTIVE DEFINITION OF DENTAL CARIES IN GRADED FORM, WITHOUT ETIOLOGICAL REFERENCE

1. Dental caries is a destructive disease of the teeth.

2. It commences on an external surface and progresses in a selectively penetrating pattern by attacking and disintegrating the several tissues comprising a tooth.

3. The resultant lesion is characterized in the enamel by a loss of translucency, superimposed discoloration, and cavitation. There occurs a distinctive lateral extension of the lesion at the dentinoenamel junction, with subsequent softening and cavitation in the dentin assuming a conical pattern with the apex toward the pulp.

4. Carious lesions developing in pits and fissures of the molars of man, the golden hamster, the white rat, and the cotton rat have a number of characteristics in common. There is the impaction of food particles into the natural occlusal crypts. In contact with this environment there is penetration of the enamel, the lateral spreading of the lesion at the juncture of the enamel and dentin, and with progression in dentin there may be a perceptible retraction of this tissue from the enamel. The disintegration of dentin to an amorphous, soft yellow to brown, leathery mass seems to be preceded by changes in the intact dentinal tubules. Spheroidal and elongated bodies considered to be bacteria, as well as certain amorphous forms, are discernible within the tubules in decreasing numbers, leading from the advancing front of the dentinal lesion toward the pulp. With further disintegration undermining the cusps and other sound tissue, secondary fracturing occurs with subsequent enlargement of the carious cavity and with eventual pulp involvement.

### MILESTONES IN CARIES RESEARCH

Since the recorded time of man, only a few really significant findings regarding dental caries have been made by investigators in different parts of the world. These constitute the biologic milestones in the field of dental caries research. Such salient observations can be epitomized best by integration into a few simple and generalized concepts.

1. As in the study of other diseases of man, investigation with animals has led to a great advance of knowledge, in this instance through experimental reproduction of carious lesions in molar teeth. The Norwegian white rat *(Rattus norvegicus albinus)*,[2-4] the Syrian hamster *(Cricetus auratus)*,[5-7] and the cotton rat *(Sigmodon hispidus hispidus)*[8, 9] all have been used successfully in many experimental dental caries studies when fed special diets. Monkeys, in smaller numbers and over longer time periods, also are now being used in such caries studies.[10, 11]
2. Evidence has accumulated showing certain microorganisms of the oral cavity to be associated with dental caries. From the depths of carious lesions as well as from tooth surfaces at the site of an initial cavity in man,[12, 13] and in experimental animals,[14-16] certain species of bacteria and, within some species, readily identifiable biochemical types of bacteria can be consistently observed. Such bacterial cells possess metabolic attributes which may be destructive to some tissues of the teeth, as shown by in vitro experiments.[17-19] Based on their laboratory cultivability, certain of these oral bacteria have been found in significantly greater numbers in mouths having considerable tooth decay, as contrasted to those free of such dental lesions.[20-22] Such a quantitative association does not necessarily constitute a cause-and-effect relationship, however.
3. That portion of the food eaten by the host but trapped upon and around the teeth constitutes the nutritive substrate for the bacterial flora of the oral cavity. When food is kept entirely out of the environment of the mouth, as has been done by stomach-tube feeding of rats, no dental caries has been observed to develop,[23] although the same diet normally ingested by rats via the usual route has been repeatedly found to be caries-productive. Further, caries incidence is markedly reduced when foodstuffs rich in refined carbohydrates are deleted from the ingested diet.[24] When all carbohydrate is eliminated from the diet, no caries developed.[25] It is common knowledge that if such refined carbohydrates as the simpler sugars constitute a part of the oral substrate, enzymatic dissimilation of these sugars can yield several organic acids. That such acids can decalcify teeth has been shown by many in vitro studies.[26, 28] Decalcification alone, however, does not represent necessarily the true nature of the whole dental caries process.
4. It has been difficult to separate the influence of heredity from the influence of various environmental factors. Yet there can be little doubt that heredity exerts an influence on the occurrence and progress of dental caries. Dependable clinical observations in man are meager, but experimental studies on some of the laboratory animals have supplied evidence bearing on this point.[29, 30] Whether hereditary influence is exerted primarily through tooth form, arrangement of the dentition, chemical composition of the enamel, dentin, and saliva, or through other yet unknown channels is still highly problematical.

5. Certain fluoride salts and other chemical substances, low in toxicity when ingested in trace amounts, have reduced the dental caries incidence in groups of experimental animals.[31-34] Most of these substances, particularly fluorides, are well-known bacterial enzyme inhibitors. It has been established presumptively that as little as one part per 1,000,000 of fluorides naturally occurring in drinking water is responsible for marked reduction in the incidence of caries in those individuals ingesting this water during the pre-eruptive development of their dentition.[35] Topical application of 2 per cent NaF to teeth in the posteruptive stage also seems to have a caries-inhibitory effect.[36, 37] The mode of action of fluorides in caries prevention is not yet fully understood. It may be the development of an enamel that is more resistant to bacterial acids or, and in addition, it may be a direct antibacterial action by the fluoride ion concentrated on a crucial site of the tooth surface.
6. Antibiotic substances which act on bacterial cells in an inhibitory or lethal manner are becoming an important factor in the study of dental caries. Observations have been made which indicate that the frequent introduction of penicillin into the oral cavity around the teeth markedly reduces the caries incidence in groups of animals,[38, 39] as well as in school children.[40] It has been reported also that other antibiotics, such as aureomycin, bacitracin, chloramphenicol, and streptomycin, which are effective in vitro in inhibiting growth of certain oral bacteria associated with dental caries,[41] likewise are useful in controlling the development of caries in teeth of hamsters and rats.[42, 43] The successful reduction of caries incidence with penicillin and, to a lesser degree, with these other antibiotics supplies strong presumptive evidence that tooth decay is in all probability a bacterial disease and, since the most effective of these antibiotic substances act primarily or selectively on gram-positive bacteria, that it is caused by gram-positive bacteria.

## USE OF GERMFREE TECHNIC

It becomes apparent from any general review of current knowledge, even as brief a summary as that in the preceding paragraphs, that the development of carious lesions in teeth is likely to be the result of convergent biologic phenomena. By this is meant that a number of factors must coexist and, since all the factors which have been identified appear to be variable ones, that these factors must all be present and active to at least a minimal, yet effective degree. For caries to develop, the resistance of the tooth must be below a certain optimal level, a nutritive substrate for microbial growth must be readily available above minimal quantities, and the presence and action of certain microorganisms are conjectured to be essential.

Along with increasing insight into the mechanisms of resistance and increased knowledge of the role of nutrition in caries prevention, there have been marked advances in the study of the bacteria of the mouth. However, since investigators have constantly been confronted with mixed flora, observations on

the role and mechanism of bacterial action have been difficult or impossible to control. Moreover, theories based on these unsatisfactory observations have been similarly difficult or impossible to prove.

In the last one-half century, several different more or less specifically identified microorganisms have been reported to be associated with the dental caries process. Those which have received more than passing attention in this regard belong to the actinomyces, cladothrix, enterococci, lactobacilli, leptotrichia, proteuslike forms, staphylococci, and streptococci. Although indirect evidence indicates that some of these bacteria are more closely associated with carious lesions than others, it is the direct evidence in experimentally produced dental caries which must be sought in order to determine the causative microbial agent or agents of this disease. Empirical methods in caries research would be supplanted by a more rational approach to the problem once an etiological agent can be irrefutably established. In vitro tests with extracted teeth composed of nonvital and, at least in some respects, altered tissues have been conducted in tubes of pure or mixed bacterial cultures. Artificial lesions sometimes have been produced, and these have been reported to resemble dental caries to a greater or lesser degree.[27, 28, 44, 45] Such in vitro experiments, valuable as they are, do not provide the comprehensive kind of direct evidence needed, and which can come only from vital teeth normally functioning within the environment of the oral cavity.

In order to provide such conditions, we have undertaken the dental caries studies in animals reared from birth under such circumstances that their mouths and teeth, as well as the rest of their bodies, their food and water, and all surfaces with which they come in contact, could be maintained completely free of all living bacteria. Under these same circumstances, a pure culture of one or more species of microorganisms may be introduced for study of their effects on the teeth in the absence of all other contaminating forms of microbial life. The idea for these very stringent circumstances has been developed by simply extending the pure culture concept practiced in microbiology to animal studies. The isolation of animals free of all other forms of life within a large, artificial enclosure which serves as a mechanical barrier to the external microbic environment initiates the germfree* animal closed system. With appropriately fabricated and interconnecting units, the isolated germ-free environment can be increased or decreased in size and complexity. This extensibility makes it mechanically feasible to provide the biologic elements essential to the life of the animals over long periods, but in the complete absence of all microorganisms. Thus, a test system (described in greater detail subsequently) has become available for study of the dental caries process in which an exact evaluation of the bacterial role at last seems possible.

The whole basic concept of "germfreeness" calls for new terms, if not for an entire system of terminology and concepts, as has been discussed by Reyniers and associates.[46]

*The single term "germfree" as used in this paper denotes that special set of circumstances or conditions in which all practical and exhaustive tests fail to demonstrate the presence of viable microorganisms. (Methods of testing are described subsequently.)

## PURPOSE

The foregoing considerations show both the necessity and the possibility for a critical experiment designed to determine whether or not germfree animals will develop lesions when fed a diet which would produce a high incidence of caries in the conventional microbe-bearing laboratory rat.* Data obtained from such an experiment using germfree rats are presented. In addition, some observations on the general state of health of the animals used also are reported. The purpose of the experiment was to compare or contrast the incidence and extent of carious lesions in the molars of conventional rats with results obtained by feeding the same sterilized diet to germfree rats.

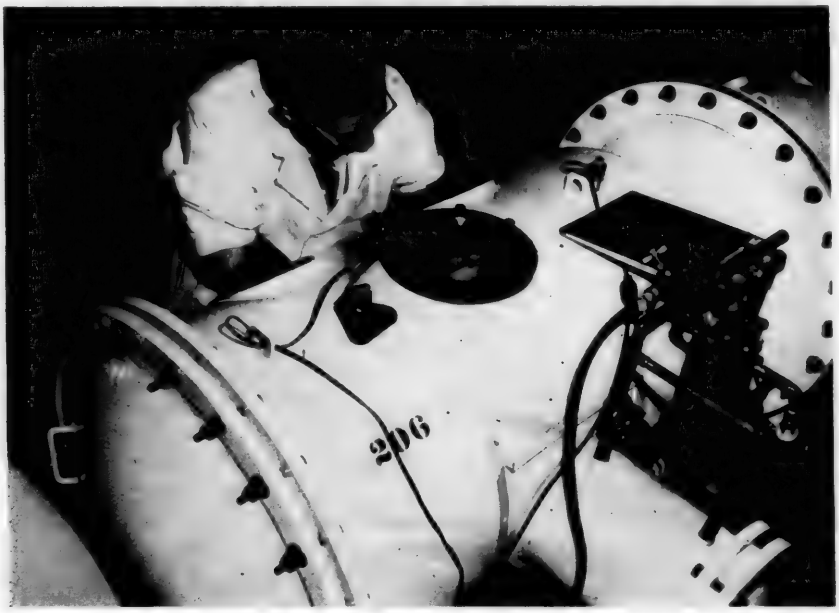


Fig. 1.—A germfree rearing unit, showing the air filter tubes and sterile lock on the right side. Through the glass viewing port is visible a white rat having its teeth examined by means of a spotlight, speculum, and exploring tine. Long rubber gloves attached to the left side of the tank permit manipulation within the germfree unit without contamination.

## EQUIPMENT, METHODS, AND MATERIALS USED

Rats from the Lobund Colony† were used throughout the study. Variation in caries susceptibility in the colony required the experimental period to be extended beyond 100 days to obtain a high and consistent caries incidence.

One group of germfree rats (1948), following cesarean birth, was hand fed through weaning (25 to 30 days) and maintained throughout the experimental period in wire-bottomed cages, while another germfree group (1951),

*Use of the expression "conventional microbe-bearing laboratory rat" emphasized the difficulty of determining precisely what constitutes a proper control for the germ-free rat. For purposes of this paper, the normal or conventional microbe-bearing animal served as a control only in so far as it consistently demonstrated the diet to be caries-productive in such rats.

†The animals comprising the Lobund Colony are inbred rats stemming from a cesarean-born, hand-fed predominantly Wistar strain.

born in naturally and suckled by germfree mother rats, was bedded on wood shavings for the concurrent purpose of breeding. Both of these groups were housed in germfree units during life. The conventional bacteria-bearing laboratory rats were taken from their mothers at weaning (21 days), one group being placed in wire-bottomed cages and the other on wood shavings in the air-conditioned colony room maintained at 22° C.

The apparatus and procedures used in obtaining cesarean-born germfree rats and then in rearing them by hand feeding have been previously reported by Reyniers, Trexler, and Ervin.[47] The type of tank used to maintain the germfree rats in this study is illustrated in Fig. 1.

*Bacteriologic Methods of Testing for Sterility.*—Sterility tests were run on a variety of samples taken from the animal itself and from various substances and surfaces within the cage. Each sample was taken on a sterile cotton swab and submitted to the bacteriologic routine either inside the cage itself or brought out to the laboratory for more detailed observation.

The samples were taken from the following sources: (1) feces and urine, (2) liquid and solid food, (3) drinking water and water used to wash and clean equipment, (4) swabbings from mouth, anus, and other orifices, (5) swabbings from fur and skin surfaces, and (6) swabbings from interior cage surfaces, such as walls, air inlet and outlet, gaskets, surfaces of the rubber gloves, and cage equipment.

Since, in these experiments, the molars were of particular interest, several samples were taken from the molar crypts and fissures by means of a fine dental broach.

For direct microscopic examination, wet mounts were prepared and observed for the presence of bacterial, fungal, and protozoan forms. Smear preparations were also made, stained by the Gram technique, and observed microscopically.

Samples were cultured on two main types of media: (1) brain-heart infusion agar enriched with 5 per cent sterile defibrinated normal horse blood and (2) fluid thioglycollate medium.* Multiple swabbings were taken from each sample source previously listed so that replicate numbers of plates of solid media and tubes of liquid media could be inoculated with each sample source to be tested. Petri plates were prepared aerobically and anaerobically and incubated at various temperatures: room temperature (approximately 25° C.), 37° C., and 50° C. Fluid thioglycollate tubes provided a gradation of oxidation-reduction potential and were incubated at the same three temperatures mentioned.

All cultures were observed periodically up to two weeks for appearance of growth. Blood agar plates were examined under X 90 magnification, and gram-stained preparations were made from thioglycollate tubes before discarding.

The data presented for the germfree rats represent results obtained from animals showing no evidence of viable microbial associates according to the test

*The solid medium was the Bacto brain-heart infusion with 1½ per cent agar added, from the Difco Laboratories, Inc., Detroit, Mich., whereas the thioglycollate medium was from the Baltimore Biological Laboratory, Inc., Baltimore, Md.

methods employed. The sterility testing procedures do not answer the difficult question of whether the animals were also virus-free. However, since virus detection depends mainly on an outward manifestation of disease or pathologic process, there was no reason to believe that the germfree groups harbored any active virus.

*Composition and Preparation of Diets.*—Of the two groups of germfree rats used, one was hand fed, the other suckled by germfree mother rats. The

TABLE I

COMPOSITION OF DIET L-128

| CONSTITUENT | AMOUNT |
|---|---|
| Casein (Labco)* | 200 G. |
| Rice (polished)† | 600 G. |
| Cellophane spangles‡ | 30 G. |
| Salts II (F-free)§ | 50 G. |
| Fat‖ | 50 G. |
| Vitamin A¶ | 8,000 IU |
| Vitamin C# | 2,000 mg. |
| Vitamin $D_3$** | 1,000 IU |
| Vitamin E†† | 500 mg. |
| Vitamin K‡‡ | 100 mg. |
| Thiamin Cl,HCl | 60 mg. |
| Riboflavin | 30 mg. |
| Pyridoxine HCl | 20 mg. |
| Ca pantothenate | 300 mg. |
| Nicotinamide | 50 mg. |
| Choline Cl | 2,000 mg. |
| Inositol | 1,000 mg. |
| p-Aminobenzoic acid | 50 mg. |
| Biotin | 1 mg. |
| Folic acid | 10 mg. |
| Nicotinic acid | 50 mg. |
| Pyridoxamine 2HCl | 4 mg. |
| $\beta$-pyracin Cl | 10 mg. |
| Yeast extract (Difco)§§ | 20 G. |
| Liver powder (Armour)‖‖ | 20 G. |
| Oil (corn)¶¶ | 16 G. |
| Starch (corn)¶¶ | 5 G. |
| Sugar water ad libitum## | |

*Vitamin-free product of The Borden Company, New York, N. Y.

†Ground and sifted to a particle size between 15 and 60 mesh.

‡Produced by Rayon Processing Co., Central Falls, R. I. Maximum particle size approaches 1 mm. in diameter.

§The composition of Lobund salts L-II in grams per kilogram of diet is: $CaCO_3$, 18.0; $CaHPO_4$, 3.3; $K_2HPO_4$, 13.5; $Na_2HPO_4$, 12.0; NaCl, 3.0; KI, 0.045; $MgSO_4 \cdot 7H_2O$, 4.5; $MnSO_4 \cdot 4H_2O$, 0.75; $Fe(C_6H_5O_7)_2$, 4.5; $CuSO_4 \cdot 5H_2O$, 0.23; $CoCl_2 \cdot 6H_2O$, 0.03; $ZnSO_4 \cdot 7H_2O$, 0.06; $Na_2B_4O_7 \cdot 10H_2O$, 0.03; and $AlK(SO_4) \cdot 10H_2O$, 0.045. All compounds were C.P. where these were available. They were mixed and stored in powdered form.

‖This hydrogenated vegetable oil is a product of the Procter & Gamble Company, Cincinnati, Ohio.

¶Vitamin A, obtained from Distillation Products, Inc., Rochester, N. Y., is a distilled natural ester form from fish liver and vegetable oils (200,000 Units per gram).

#Ascorbic acid and the B-vitamins were the crystalline products obtained from commercial sources.

**Vitamin D is Delsterol, obtained through the courtesy of Dr. J. Waddell at E. I. du Pont de Nemours and Co., Newark, Del. It is irradiated 7-dehydrocholesterol (200,000 A.O.A.C. Units per gram) in vegetable oil.

††Vitamin E is $\alpha$-tocopherol from Merck and Company, Inc., Rahway, N. J.

‡‡Vitamin K is 2-me-1, 4-naphthoquinone from Merck and Company, Inc.

§§Difco Laboratories, Inc., Detroit, Mich.

‖‖Whole dried pork liver powder produced by Armour Laboratories, Chicago, Ill.

¶¶Produced by the Corn Refining Co., Argo, Ill.

##This is 5 per cent sucrose, obtained from local stores, dissolved in distilled water.

hand-fed rats were given sterile liquid diets similar to diet 24N, which has been described.[47] Sodium fluoride was omitted from the salt mix since it was desirable to keep diets as low in fluorides as practical. Solid-food diet (L-128) was placed before these rats as soon as their eyes were opened. They generally ate very little of it before they were 16 to 18 days old.

Conventional control rats also received rat milk from the mother until weaned. They began nibbling at about 12 to 14 days of age on some of the stock diet provided for the mother rat.*

All rats during the experimental period received diet L-128 sterilized by steam at seventeen pounds per square inch for twenty-five minutes (Table I). Some of the rats were allowed to drink a 5 per cent sucrose solution. The rats apparently liked their sugar water (daily consumption of about 95 ml. sucrose solution and 12 Gm. diet in 150 to 200 Gm. rats); thus, about 30 per cent of the diet by weight was sucrose.

The average fluoride content in parts per million of the diets (three to five determinations) is: Diet L-128, 2.12; stock diet, 16.88; milk formulae, 2.91; and sugar water, 0.58.

*Dental Tissue Preparation.*—After the animals were sacrificed with ether, the tissues of the whole body were carefully observed and evaluated. The head was detached and prepared by the following routine procedures: The skin was removed and the head was cleaved on the longitudinal median line into two sagittal sections. These were placed into a jar containing 10 per cent neutral formalin for forty-eight hours; after a washing in water, 70 per cent ethanol replaced the formalin in the storage jar.

To provide a growth record of the rat skull at this stage of preparation, before further dissection, a roentgenogram was taken of both halves of all heads. This procedure consisted of setting the molar teeth in occlusion in the left hemiskull, while in the right, the maxillary and mandibular molars were separated and fixed out of occlusion. These positions were maintained during the roentgenographing procedures with a resinous wax which proved to be radiolucent. An exposure of three-fourths second on a 40 kv. dental x-ray machine equipped with a twenty-inch target-object distance provided 9.75 milliampere seconds and resulted in clear roentgenograms having optimal density. After processing and labeling, these negative films were employed in determining the several craniometric distances used in skull growth evaluation (Fig. 2).

The next procedure was to dissect the skull, removing the quadrants of molars for gross dental caries observation. Each quadrant, containing three molars, was magnified seven, fifteen, or twenty times under a binocular dissecting microscope. Using a microair blast device consisting of a No. 27 gauge hypodermic needle locked in heavy rubber tubing connected to a source of compressed air, the dental area of each specimen was dried and freed of loose debris for better observation of the teeth. A micro-lancet prepared from a large dissecting needle was used to perform a complete gingivectomy. With a barbed

*The stock diet for the Lobund Colony is commercially prepared Rockland Rat Diet from the Arcady Farms Milling Co., Chicago, Ill.

dental root canal broach, the larger food particles wedged into the occlusal portion of the molar sulci could be removed intact. Employing finer broaches and picks with extreme care, more foreign particles could be dislodged farther down in the natural molar crevices. Finally, a very fine smooth root canal broach was used to explore for carious lesions by tactile and visual means the depths of all pits, fissures, and sulci, as well as all smooth surfaces of each molar.

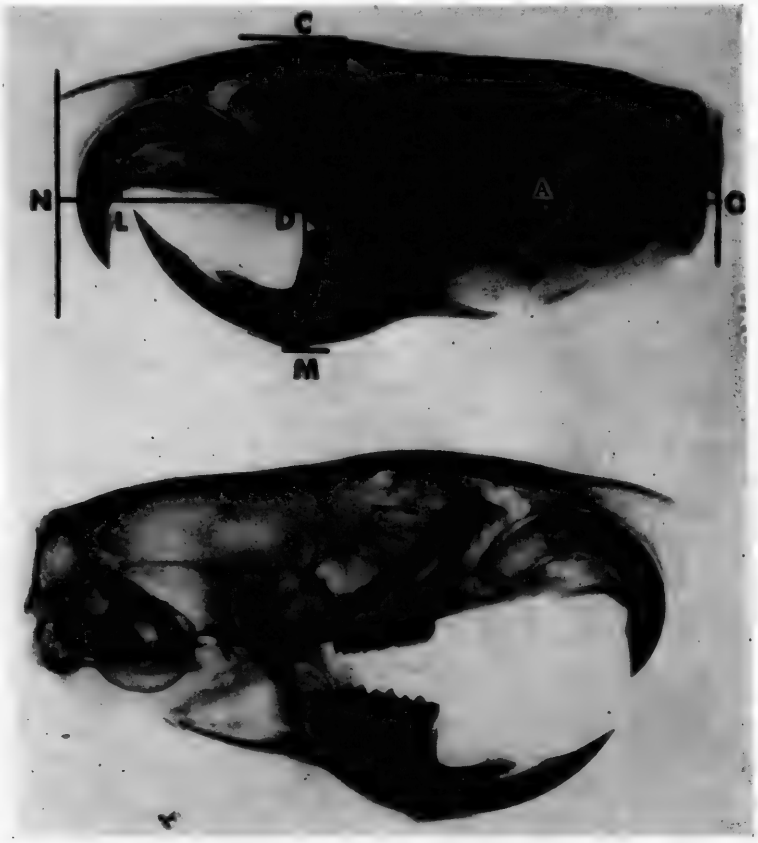



Fig. 2.—Roentgenogram positive of the two halves of a rat's skull, indicating the established craniometric distances on the left half.

For caries observations under higher magnification, ground sections and surfaces were prepared. This was done by embedding in a clear plastic (methyl methacrylate) certain quadrants of the dentition. Each quadrant was arranged within the confines of a heavy brass flask so that the buccal surfaces of the molars would face one edge of the square plastic briquet after processing. Each lateral surface of the briquet could then be ground to the level of the molar teeth on a specially constructed power-driven grinding apparatus. By alternate grinding and observing under the microscope at every 0.2 mm. depth of tooth structure, a sequential view of each molar in its entirety could be gained.

Decalcified sections of the molars were also prepared whenever indicated. Following decalcification in equal parts of 45 per cent formic acid and 20 per cent sodium citrate for four days, all the tissues in a quadrant were embedded in celloidin and sectioned on the microtome. Stained serial sections, particularly those stained to depict bacteria by the Brown and Brenn method,[48] were observed for lesions of dental caries.

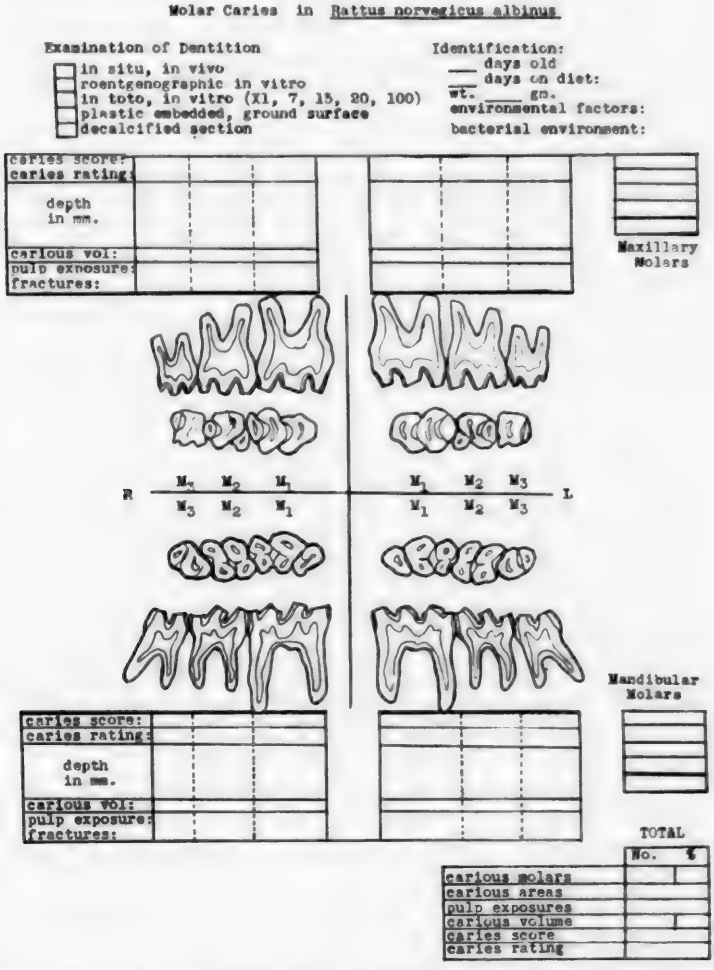



Fig. 3.—Chart used in comprehensively evaluating rat molar caries. The qualitative and quantitative determination of dental caries was carried out by recording on the chart in red the exact location and extent of every carious lesion, whether observed macroscopically in situ or microscopically in ground or decalcified sections.

*Caries Evaluation Procedure.*—In the literature of the past two decades, a variety of methods for scoring carious lesions in the rat have been reported.[3, 4, 29, 49, 50] So that the observations recorded in this study might be equilibrated

TABLE II

GROWTH AND DEVELOPMENT OF RATS

| NO. OF ANIMALS | | SEX | TOTAL AGE (DAYS) | DAYS ON EXPERIMENT | SKULL MEASUREMENTS FROM CEPHALOROENTGENOGRAMS (MM.) O-N | C-M | D-L | WEIGHT AT END OF EXPERIMENT (GM.) | LENGTH OF FEMUR (MM.) | F-CONTENT OF FEMUR (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|
| *Conventional Animals of 1948* | | | | | | | | | | |
| Average | 7 | ♂♂ | 160 | 139 | 48.0 | 22.2 | 14.1 | 447 | 33.1 | 42.1 |
| Range | | | 158-164 | 137-143 | 46.9-49.0 | 21.4-22-8 | 13.8-15.0 | 374-501 | 32.0-33.8 | 35.4-48.3 |
| Average | 9 | ♀♀ | 160 | 139 | 44.0 | 20.7 | 12.9 | 254 | 28.5 | 54.5 |
| Range | | | 158-164 | 137-143 | 43.0-45.6 | 20.0-21.5 | 12.2-13.3 | 224-272 | 27.4-29.7 | 47.3-66.6 |
| Group average | | | 160 | 139 | 45.8 | 21.4 | 13.4 | 338 | 30.6 | 48.8 |
| *Germfree Animals (Hand fed) of 1948* | | | | | | | | | | |
| Average | 7 | ♂♂ | 167 | 141 | 42.7 | 20.0 | 12.4 | 325 | 20.1 | 63.7 |
| Range | | | 163-179 | 137-153 | 41.7-43.4 | 19.4-21.0 | 12.0-13.0 | 285-368 | 19.2-22.8 | 45.6-76.1 |
| Average | 6 | ♀♀ | 170 | 145 | 38.8 | 18.4 | 11.2 | 229 | 18.1 | 72.3 |
| Range | | | 163-179 | 137-153 | 37.5-40.8 | 17.9-19.3 | 10.8-11.7 | 145-332 | 16.5-19.9 | 56.0-83.0 |
| Group average | | | 168 | 143 | 40.9 | 19.3 | 11.8 | 281 | 19.4 | 67.6 |
| *Conventional Animals of 1951* | | | | | | | | | | |
| Average | 10 | ♂♂ | 191 | 170 | 48.0 | 23.4 | 13.7 | 431 | 37.1 | 53.7 |
| Range | | | 162-260 | 141-240 | 45.0-50.0 | 22.5-24.4 | 11.8-14.3 | 395-530 | 35.8-39.1 | 22.8-78.3 |
| Average | 13 | ♀♀ | 199 | 178 | 44.3 | 21.3 | 12.7 | 249 | 32.0 | 66.3 |
| Range | | | 162-260 | 141-240 | 42.5-47.3 | 20.0-22.3 | 12.0-13.3 | 215-295 | 29.0-34.6 | 32.3-104.8 |
| Group average | | | 195 | 174 | 45.9 | 22.2 | 13.2 | 328 | 34.3 | 60.6 |
| *Germfree Animals (Dam fed) of 1951* | | | | | | | | | | |
| Average | 3 | ♂♂ | 193 | 173 | 45.9 | 22.3 | 12.8 | 313 | | |
| Range | | | 162-256 | 142-236 | 44.3-48.9 | 22.0-23.0 | 12.5-13.3 | 308-322 | | |
| Average | 6 | ♀♀ | 225 | 205 | 44.1 | 21.1 | 12.0 | 219 | No data on femurs | |
| Range | | | 162-256 | 142-236 | 42.5-46.0 | 20.5-21.8 | 11.0-12.8 | 192-235 | | |
| Group average | | | 214 | 194 | 44.7 | 21.5 | 12.3 | 250 | | |

as far as possible with data reported in other studies, the molar teeth of rats observed in this project were evaluated by the following eight methods: (1) An individual rat was considered to be positive for gross caries if 1 or more molar teeth (in toto) showed obvious carious lesions. (2) A numerical score was assigned to all gross carious lesions in susceptible areas when observed in toto and under magnifications up to fifteen or twenty diameters. This score included not only number, but depth and extent of lesions as well. (Method of Cox, Dodds, Dixon, and Matuschak.[49]) (3) The absolute number of carious molars per rat was determined by microscopic observation of ground and sectioned teeth. (4) The percentage of carious molars in a rat was determined by calculation from the data supplied by the previous method. (5) The absolute number of carious areas per rat was based on microscopic observation of ground and sectioned teeth. (6) A caries rating of fissure lesions was based on microscopic observation of ground and sectioned teeth. (Method of Rosebury, Karshan, and Foley.[50]) (7) An approximated volumetric evaluation of tissue destroyed by caries was determined by microscopic observation of ground and sectioned teeth. (8) The percentage of caries-destroyed tissue on a volumetric basis relative to the average total volume of tooth structure comprising the anatomic crowns was determined by calculation from data supplied by the previous method. The chart used throughout the study for recording rat caries by these multiple methods is illustrated in Fig. 3.

## RESULTS

In order that any major differences in growth and development of the germfree and conventional control animals might be observed and recorded, comparisons were made of weight, skull dimensions, femur length, and general health.

*Weight.*—Preliminary studies had indicated that the diet was nutritionally adequate (the diet had been steam sterilized and fed to rats through four generations before it was accepted for germfree work). The growth of the germfree and conventional rats is indicated by their weights taken immediately before sacrifice (Table II). As expected, based on earlier observations by Reyniers, Trexler, and Ervin,[47] the hand-fed germfree rats were not as heavy at weaning as the dam-reared animals. However, the postweaning growth of these rats is equal to that of the stock rats. The pertinent point is that these animals all grew at acceptable rates for use in a dental caries study.

*Craniometry.*—Measurements were made of the distances between certain cranial landmarks on the roentgenogram of the rat hemiskull. Craniometric distances selected as indicative of the skull growth pattern were those measured by Spence.[51] These distances, illustrated in Fig. 2, were from the occipital to the nasal point (*O-N*); from the most superior to the most inferior skeletal registration when measured along a perpendicular erected on *O-N* at the mesial surface of the lower first molar (*C-M*); and from the most anterior portion of the maxillary first molar to the lingual surface of the maxillary incisor, which thus constituted the diastema (*D-L*).

Data compiled in Table II show the average values in millimeters of O-N, C-M, and D-L, respectively, of one (1948) conventional group of rats to be 48.0, 22.2, and 14.1 for the males and 44.0, 20.7, and 12.9 for the females. Animals comprising a later (1951) conventional group measured, respectively, 48.0, 23.4, and 13.7 for males and 44.3, 21.3, and 12.7 for females. These craniometric distances can be compared with those of the (1948) germfree group of rats born by cesarean section and hand fed, which averaged 42.7, 20.0, and 12.4 for males and 38.8, 18.4, and 11.2 for females, while those born germfree and suckled by their germfree mothers (1951) had skull measurements of 45.9, 22.3, and 12.8 for males and 44.1, 21.1, and 12.0 for females. These figures indicate that the composite skull dimensions of the conventional bacteria-bearing animals exceeded those of the hand-fed germfree rats by 11.1 per cent and of the suckled germfree rats by 3.0 per cent.

*Femur Lengths.*—Whenever available, femurs of the rats also were measured as an index of growth and development.

*General Health.*—The germfree rat has been described in some detail morphologically and functionally by Reyniers and Trexler,[52] Reyniers, Trexler, and Ervin,[47] and Gustafsson.[53] These descriptions were based on complete autopsy, hematologic study, organ weight, and gross biochemical analyses. The publication of a systematic survey of the germfree rat is planned in a future Lobund report.

For general comparative purposes, it can be stated that the cesarean-born, hand-reared germfree rat presents in the course of its life an identical pattern to the comparable conventional animal. The only conspicuous differences observed in the hand-fed germfree rats included in this report have been retarded growth during the preweaning period and occasionally expanded caeca and lens cataracts. No such differences have been observed, however, in germfree rats suckled by a germfree mother rat. Furthermore, as an indication of normal metabolism in all rats studied, neither the germfree nor the conventional groups showed any evidence of retarded eruption or disturbance in calcification of their molar or incisor teeth.

A low fluoride content of teeth and certain bones was considered to be indicative of the minimal intake of fluorides in the diet fed. The average femur contained only 59.4 ppm of the fluoride ion, as contrasted with 223.9 ppm in rats of the Lobund Colony, consuming the commercially prepared stock diet.

*Comparative Data on Dental Caries in Germfree and Conventional Animals.*—The end point of this study is the evaluation of amount and type of carious destruction which occurs, if any, under the germfree conditions established. In this kind of study, wherein there is maintained complete microbial control of the animals and their immediate environment, the presence of even small carious lesions would be indicative of great significance. On the other hand, mere size of a lesion, at least when within the same dental tissue, is of only quantitative importance and is usually related to the factor of time. Therefore, to be assured of the presence or absence of even the smallest microscopic lesion, sectioning of all molar teeth and microscopic examination of every component appeared mandatory, and was accomplished.

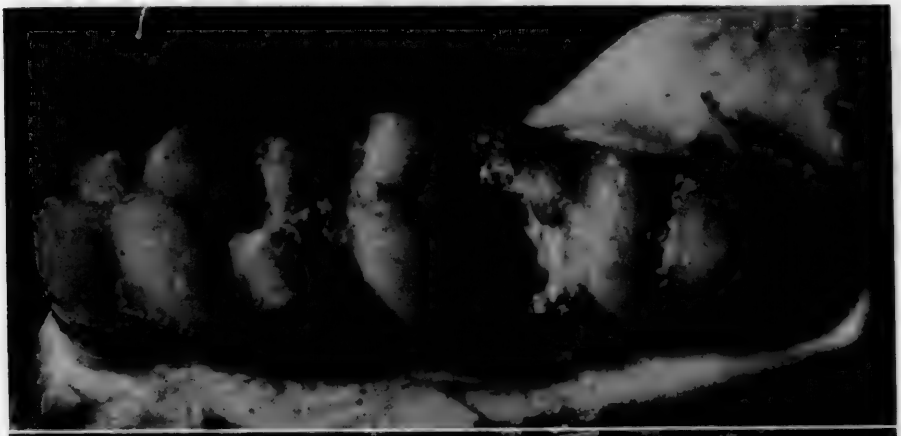


Fig. 4.

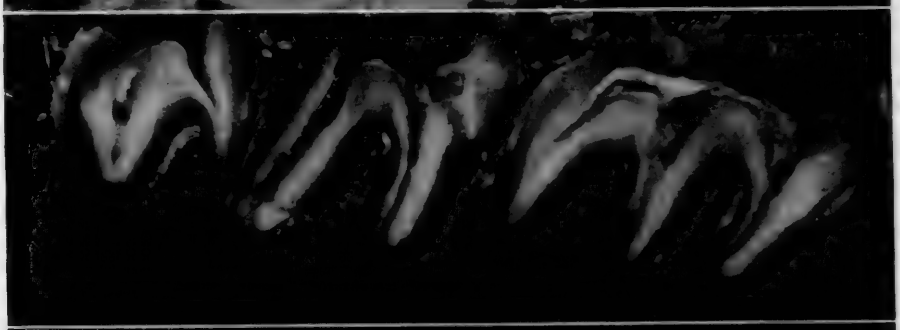


Fig. 5.

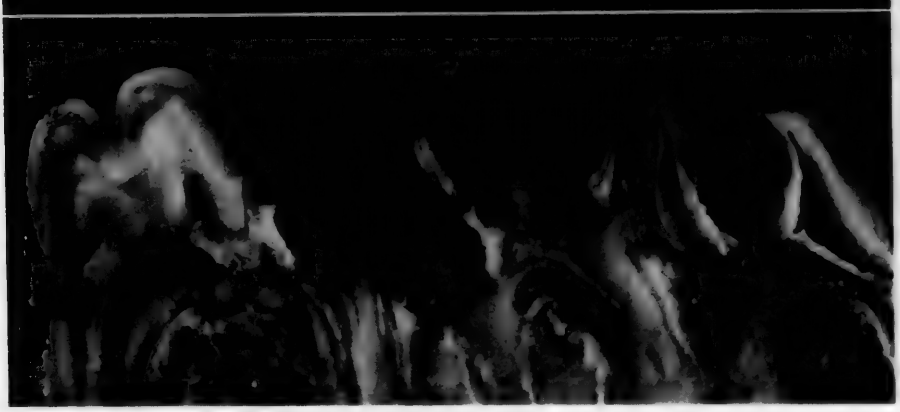


Fig. 6.

Conventional control rats, showing representative carious lesions.

Fig. 4.—Lower right quadrant of molars (Rat 18LOB2-36 No. 2♀). There is extensive carious destruction of the second molar and a smaller lesion in the first molar. The third molar, as in this instance, rarely shows any lesions. (Magnification ×18.)

Fig. 5.—Ground surface of upper right molars (Rat 18LOB2-45 No. 10♀) photographed by reflected light from the buccal aspect. These molars show no gross carious destruction, as is usually true for maxillary molars. (Magnification ×18.)

Fig. 6.—Ground surface of lower left molars as viewed from the buccal (Rat 55N1-1B No. 24♀). Two large carious lesions involving the pulps are visible in the first and second molars. (Magnification ×18.)

Fig. 7.

Fig. 8.

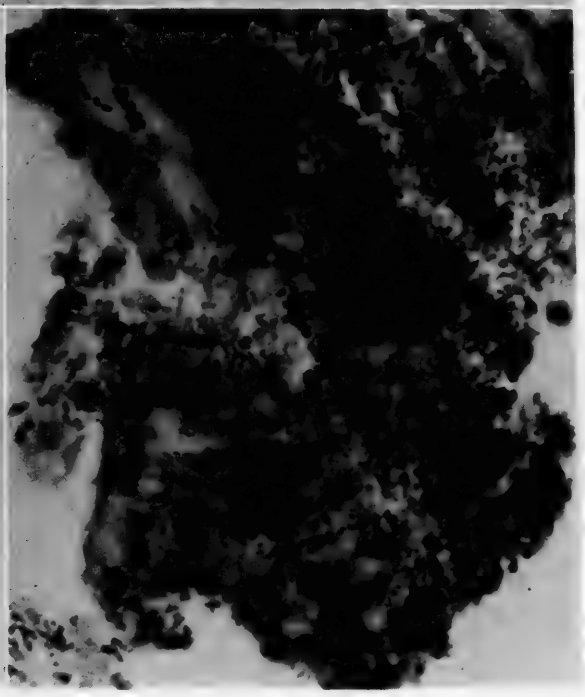


Fig. 9.

Conventional control rats, showing representative carious lesions.

Fig. 7.—A decalcified section, Brown and Brenn stained, of the upper right molars as seen from the lingual (Rat 18LOB2-45 No. 5♂). In the second molar, a small dentinal lesion is apparent on the mesial wall of the central sulcus. A large penetrating lesion has caused considerable destruction in the first molar. (Magnification ×18.)

Fig. 8.—Magnified dentin in the central area of the first molar, pictured in Fig. 7, being invaded and apparently disintegrated by bacteria. (Magnification ×330.)

Fig. 9.—Greater magnification of dentin fragment, seen in Fig. 8, showing the bacteria-laden tissue. Microorganisms appear to be of mixed morphologic types. (Magnification ×1,100.)

Fig. 10.

Fig. 11.

Fig. 12.

Conventional control rats, showing representative carious lesions.

Fig. 10.—Lingual view of lower right quadrant depicting 3 decalcified molars (Rat 18LOB2-45 No. 7♀). There is apparent bacterial invasion of the second molar dentin, but extensive carious destruction in the first molar. (Magnification ×18.)

Fig. 11.—Enlarged view of disintegrating cuspal wall of first molar pictured in Fig. 10. Note backward penetration of dentinal tubules. A lamella leading from this food-packed mesial sulcus is also noteworthy. (Magnification ×330.)

Fig. 12.—High-power magnification of dentin seen in Fig. 11. Bacterial cells and microcolonies are very plentiful in this final stage of dentin disintegration. (Magnification ×1,100.)

Since a number of methods have been utilized by other investigators in scoring experimental dental caries in the rat, it was deemed logical to equilibrate the observations made in this study with those reported in the past. Thus, multiple methods of evaluation were drawn upon. Surface examination of the molar teeth of the dietary control rats whose bacterial flora remained uncontrolled showed carious lesions to be present in almost every one of the thirty-nine (Figs. 4 to 12 and Tables III and IV). The average numerical score per rat for these lesions, based on the susceptible area concept and considering both depth and extent of the gross lesion, was 21.5. Sectioning on the microtome and grinding the plastic embedded teeth of these same animals permitted ultimate microscopic evaluation of the total number and percentage of molars actually carious. There was an average of four carious molars per rat, which was 33.3 per cent

TABLE III

DENTAL CARIES EVALUATION

| IDENTIFICATION NUMBER | MICROBIAL ENVIRONMENT | MACROSCOPIC OBSERVATION OF MOLARS IN TOTO | | MICROSCOPIC OBSERVATION OF SECTIONED SPECIMENS | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | CARIOUS MOLARS | | NUMBER OF CARIOUS AREAS | CARIES RATING | VOLUMETRIC CARIOUS DESTRUCTION | |
| | | GROSS CARIES | GROSS CARIES SCORE | NUMBER | PER CENT | | | CUBIC M.M. | PER CENT OF TOTAL CROWNS |
| 18LOB2-36 #1 ♂ | Uncontrolled | + | 7 | 4 | 33.3 | 4 | 11 | 0.08 | 0.2 |
| 18LOB2-36 #6 ♂ | | + | 4 | 4 | 33.3 | 4 | 15 | 0.10 | 0.3 |
| 18LOB2-45 #1 ♂ | | + | 29 | 4 | 33.3 | 4 | 42 | 5.80 | 16.1 |
| 18LOB2-45 #2 ♂ | | + | 53 | 4 | 33.3 | 4 | 42 | 12.50 | 34.7 |
| 18LOB2-45 #3 ♂ | | + | 51 | 5 | 41.7 | 5 | 50 | 12.90 | 35.8 |
| 18LOB2-45 #4 ♂ | | + | 37 | 4 | 33.3 | 4 | 42 | 7.34 | 20.4 |
| 18LOB2-45 #5 ♂ | | + | 45 | 8 | 66.7 | 8 | 56 | 5.76 | 16.0 |
| 18LOB2-36 #2 ♀ | | + | 21 | 5 | 41.7 | 5 | 38 | 2.39 | 6.6 |
| 18LOB2-36 #3 ♀ | | + | 14 | 4 | 33.3 | 4 | 28 | 0.70 | 1.9 |
| 18LOB2-36 #4 ♀ | | + | 23 | 4 | 33.3 | 4 | 35 | 1.83 | 5.1 |
| 18LOB2-45 #7 ♀ | | + | 23 | 6 | 50.0 | 8 | 38 | 4.58 | 12.7 |
| 18LOB2-45 #8 ♀ | | + | 25 | 5 | 41.7 | 6 | 40 | 7.58 | 21.1 |
| 18LOB2-45 #9 ♀ | | + | 53 | 4 | 33.3 | 4 | 44 | 12.68 | 35.2 |
| 18LOB2-45 #10 ♀ | | + | 36 | 5 | 41.7 | 5 | 43 | 5.60 | 15.6 |
| 18LOB2-45 #11 ♀ | | + | 30 | 5 | 41.7 | 5 | 44 | 3.20 | 8.9 |
| 18LOB2-45 #12 ♀ | | + | 34 | 5 | 41.7 | 5 | 44 | 4.34 | 12.1 |
| Average | | + | 30.3 | 4.8 | 39.6 | 4.9 | 38.3 | 5.46 | 15.2 |
| 3LOB2-36 #2 ♂ | Germfree† | –* | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-1A #3 ♂ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-2A #5 ♂ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-2A #6 ♂ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-2A #7 ♂ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-2A #8 ♂ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 3LOB2-36 #1 ♀ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-1A #1 ♀ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-1A #2 ♀ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-2A #1 ♀ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-2A #2 ♀ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-2A #3 ♀ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25LZC2-2A #4 ♂ | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| Average | | – | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |

*One molar found fractured.

†These rats were cesarean born and were hand fed until of weaning age.

of the twelve molars present in the oral cavity. The absolute number of individual carious areas averaged 4.3 per rat. For these same conventional animals, a calculated volumetric evaluation of dental tissue actually destroyed by caries was also determined. This was done by measuring the carious area in each section or ground surface with a micrometer disk set in the microscope eyepiece, and then multiplying the size of these carious areas by the degree of penetration, represented by the number of consecutive sections or surfaces involved. The volume of tooth substance destroyed average 3.54 cubic millimeters per rat. The

TABLE IV
DENTAL CARIES EVALUATION

| IDENTIFICATION NUMBER* | MICROBIAL ENVIRONMENT | MACROSCOPIC OBSERVATION OF MOLARS IN TOTO | | MICROSCOPIC OBSERVATION OF SECTIONED SPECIMENS | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | CARIOUS MOLARS | | NUMBER OF CARIOUS AREAS | CARIES RATING | VOLUMETRIC CARIOUS DESTRUCTION | |
| | | GROSS CARIES | GROSS CARIES SCORE | NUMBER | PER CENT | | | CUBIC MM. | PER CENT OF TOTAL CROWNS |
| 55N1-1B #1 ♂ | Uncontrolled | + | 3 | 2 | 16.7 | 3 | 4 | 0.02 | 0.1 |
| 55N1-1B #2 ♂ | | + | 2 | 2 | 16.7 | 2 | 11 | 0.08 | 0.2 |
| 55N1-1B #6 ♂ | | + | 2 | 2 | 16.7 | 3 | 9 | 0.10 | 0.3 |
| 55N1-1B #8 ♂ | | − | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 55N1-1B #10 ♂ | | + | 8 | 4 | 33.3 | 5 | 18 | 0.42 | 1.2 |
| 55N1-1B #11 ♂ | | − | 0 | 1 | 8.3 | 1 | 2 | 0.004 | 0.01 |
| 55N1-1B #12 ♂ | | + | 4 | 2 | 16.7 | 2 | 8 | 0.16 | 0.4 |
| 55N1-1B #27 ♂ | | + | 26 | 4 | 33.3 | 5 | 41 | 3.17 | 8.8 |
| 55N1-1B #28 ♂ | | +† | 10 | 3 | 25.0 | 3 | 20 | 0.62 | 1.7 |
| 55N1-1B #31 ♂ | | + | 31 | 5 | 41.7 | 6 | 45 | 6.43 | 17.8 |
| 55N1-1B #3 ♀ | | + | 3 | 2 | 16.7 | 2 | 9 | 0.08 | 0.2 |
| 55N1-1B #4 ♀ | | + | 3 | 3 | 25.0 | 4 | 7 | 0.11 | 0.3 |
| 55N1-1B #5 ♀ | | + | 8 | 5 | 41.7 | 6 | 19 | 0.45 | 1.2 |
| 55N1-1B #7 ♀ | | +† | 2 | 2 | 16.7 | 3 | 5 | 0.02 | 0.1 |
| 55N1-1B #9 ♀ | | + | 7 | 3 | 25.0 | 4 | 7 | 0.14 | 0.4 |
| 55N1-1B #13 ♀ | | + | 23 | 6 | 50.0 | 7 | 23 | 3.99 | 11.1 |
| 55N1-1B #14 ♀ | | + | 19 | 4 | 33.3 | 5 | 21 | 0.44 | 1.2 |
| 55N1-1B #15 ♀ | | − | 0 | 1 | 8.3 | 1 | 2 | 0.02 | 0.1 |
| 55N1-1B #24 ♀ | | + | 34 | 4 | 33.3 | 5 | 46 | 4.49 | 12.4 |
| 55N1-1B #25 ♀ | | + | 17 | 4 | 33.3 | 4 | 30 | 0.60 | 1.6 |
| 55N1-1B #26 ♀ | | + | 49 | 5 | 41.7 | 5 | 57 | 7.60 | 21.1 |
| 55N1-1B #29 ♀ | | + | 27 | 4 | 33.3 | 4 | 33 | 3.02 | 8.3 |
| 55N1-1B #30 ♀ | | + | 15 | 5 | 41.7 | 5 | 28 | 5.45 | 15.1 |
| Average | | + | 12.7 | 3.2 | 26.5 | 3.7 | 19.3 | 1.62 | 4.5 |
| 25J1-1 #3-1-1 ♂ | Germfree‡ | − | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25J1-1 #3-1-4 ♂ | | − | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25J1-1 #2-2-8 ♂ | | −† | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25J1-1 #3-1-2 ♀ | | − | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25J1-1 #3-1-3 ♀ | | −† | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25J1-1 #2-2-1 ♀ | | −† | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25J1-1 #2-2-2 ♀ | | − | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25J1-1 #2-2-5 ♀ | | − | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| 25J1-1 #2-2-6 ♀ | | − | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |
| Average | | − | 0 | 0 | 0.0 | 0 | 0 | 0.00 | 0.0 |

*All these animals (of 1951), in contradistinction to those (of 1948) listed in Table III, were bedded on wood shavings and received no sucrose water.

†A molar cusp found fractured.

‡These rats were born and suckled by germfree mother rats.

Fig. 13.

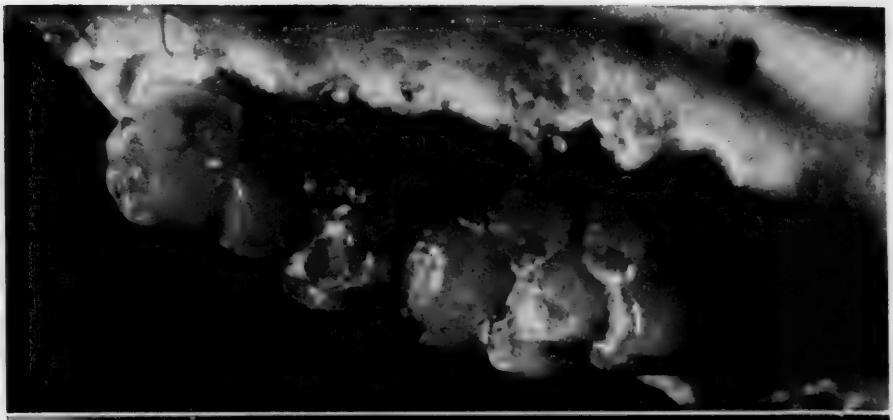
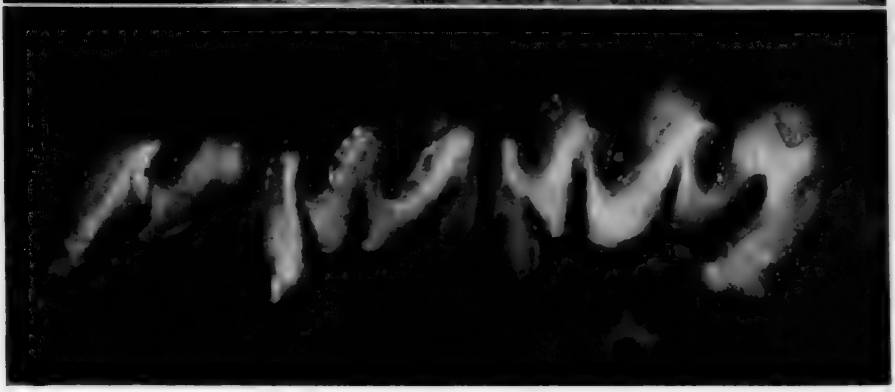


Fig. 14.

Fig. 15.



Germfree rats.

Fig. 13.—Upper left molars (Rat 25LZC2-1A No. 2♀) showing no caries, but some degree of occulsal wear. (Magnification ×18.)

Fig. 14.—Lower left molars (Rat 3LOB2-36 No. 2♂). No carious lesions were found and very little staining of tooth surfaces was observed. (Magnification ×18.)

Fig. 15.—Ground surface of the lower left three molars as seen from the lingual aspect (Rat 25LZC2-A2 No. 3♀). No caries was observed in the enamel or dentin. (Magnification ×18.)

Fig. 16.

Fig. 17.

Fig. 18.

Fig. 19.

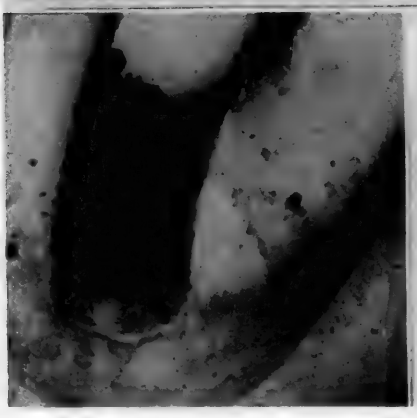
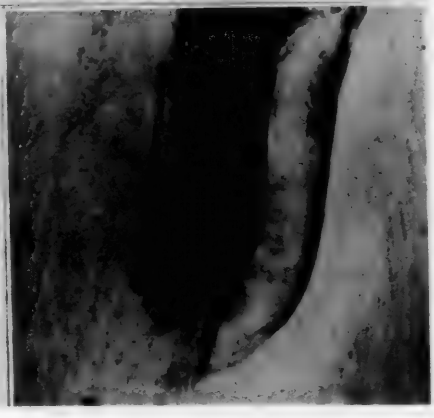


Fig. 20.



Germfree rats.

Fig. 16.—Ground section of the lower right molars as viewed from the buccal aspect and photographed by transmitted light (Rat 25LZC2-2A No. 7♂). No carious lesions were apparent. (Magnification ×18.)

Fig. 17.—Enlarged view of the second molar central sulcus and mesial cusp seen in Fig. 16. Note pattern of abrasion, the less resistant dentin wearing away earlier than the surrounding enamel. (Magnification ×50.)

Fig. 18.—Higher magnification of the sulcus seen in Fig. 17. Note intact enamel wall and impacted food debris. (Magnification ×160.)

Fig. 19.—First molar cusp and mesial sulcus seen in Fig. 16. (Magnification ×50.)

Fig. 20.—Enlarged view of the sulcus with lamellae seen in Fig. 19. (Magnification ×160.)

Fig. 21.

Fig. 22.



Fig. 23.



Fig. 21.—Stained decalcified section of lower left first molar as seen from the lingual (Rat 25LZC2-2A No. 2♀). (Magnification ×70.)

Fig. 22.—Greater magnification of dentin in first molar central sulcus seen in Fig. 21. No carious dentin was detected. (Magnification ×330.)

Fig. 23.—Very high magnification of section seen in Fig. 22. Note intact, ramifying dentinal tubules. (Magnification ×1,100.)

average total volume of the coronal portion of twelve rat molars is approximately 36 cubic millimeters, and thus 9.9 per cent by volume of all molar crowns were observed to be disintegrated by the carious process. The numerical rating scale employed to evaluate the incidence and magnitude of fissure caries in these sectioned molars indicated an average rating index of 28.8 per rat. This scale was based on the various microscopic stages of caries observed and first described by Rosebury, Karshan, and Foley,[50] and later by Schamp and Leicester.[54] It should be noted that those conventional rats, listed in Table IV, bedded on pine wood shavings and receiving plain sterile distilled water as a group had much less caries than those, listed in Table III, without such bedding material and ingesting the 5 per cent sucrose solution as their sole water supply.

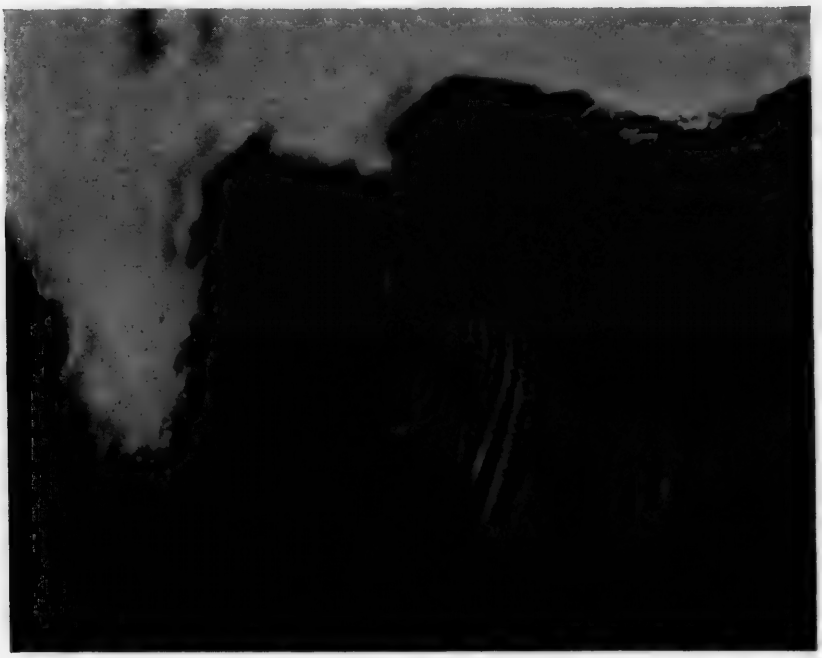


Fig. 24.—Germfree rat. Decalcified section of fractured edge of lower right first molar (Rat 3LOB2-36 No. 2♂). (Magnification ×2,500.) The fractured molar was found in this germfree animal on sacrifice. Note sharp fracture lines and lack of disintegration as contrasted to typical carious dentin seen in Figs. 8, 9, 11, and 12.

In marked contrast to these conventional rats with their usual microbial flora, the twenty-two rats reared and maintained entirely free of discernible microbial life, but ingesting the same diet, showed the complete absence of any carious lesions, as determined by the same microscopic methods just described (Figs. 13 to 23 and Tables III and IV). There were observed, however, in this group of germfree rats, four molars where a cusp or some other coronal aspect had been definitely fractured, probably on a traumatic basis, yet showing no evidence of caries (Fig. 24). Other observations of the germfree rats indicated

that their molars were normally developed morphologically and showed normal occlusal wear of the cuspal areas, but seemed, as a group, much cleaner than the teeth of the conventional control animals (Figs. 13 and 14).

## DISCUSSION

Knowledge of the dental caries process has been advanced considerably by the use of animals in whose dentition it has been possible to induce this disease on an experimental basis. The white rat was the first rodent to be utilized in such studies, and repeatedly has demonstrated its usefulness in various kinds of experiments concerned with carious lesions developing in its molar teeth. The morphologic pattern of rat molars is such as to provide areas of food entrapment very similar to the pits and fissures found normally in the molars and premolars of man. There also have been other animals utilized in experimental caries studies, notably, the golden hamster and the cotton rat. These rodents may be more suitable than the common laboratory rat for observation of certain aspects of this dental disease. However, for the study reported here, centered as it is on the use of the germfree technic, it was almost imperative that the white Norwegian rat *(rattus norvegicus albinus)* be employed for the practical reason that it was the animal best understood biologically, and therefore most easily reared under the exacting conditions of germfree life. Moreover, the particular strain used had been previously demonstrated to be caries-susceptible. It is possible experimentally, as has been shown herein, to rear germfree rats from a strain which satisfied most of the experimental criteria and to show that caries is not produced in a reasonable test period. This experiment, while critical, lies within a framework of limitations and does not answer the problem of etiology. It simply and positively shows that dental caries is not produced in a pure rat system, that is, in the absence of microbes. It leaves unexplored the mechanism of dental caries and, except in a negative sense, the etiology of the disease. However, it is apparent from the observations here reported that the necessary steps toward the pure culture approach have been experimentally implemented.

Since dental caries is in all probability a convergent biologic phenomenon in which there must be the coincidental occurrence of at least three conditions, the use of the germfree animal in this study permitted complete control over the most difficult factor, the ubiquitous microbial flora. Another factor, the presence of a nutritive substrate metabolized by bacteria of the oral cavity, has been within experimental control for some time. The third and most intrinsic factor, a suboptimal resistance of the tooth itself, is only incompletely understood. Tooth resistance may be concerned with the presence of trace elements incorporated in the enamel and dentin. Bearing on this point, the fluoride ion content was held to a minimum in both liquid and solid components of all diets fed the animals. General tooth resistance might also involve a certain self-cleansing type of dental morphology or possibly other less tangible factors capable of assertion on a genetic basis.

Complete control of microorganisms living in and about the experimental animal by the germfree technic has made it possible in the present basic study to exclude all bacteria. It is notable that the same diet, fed to rats under normal conditions of the animal room where it produced abundant dental caries, failed to produce a single carious lesion, even on a microscopic level, in the germfree rats. Thirteen germfree rats included in this report, it is true, were born by cesarean section and hand fed a sterile, synthetic milk diet, unlike the conventional control animals, which were normally born and suckled by the mother rat. Yet the mechanics of the germfree method, indispensable in the initial stages of germfree rearing and difficult as it is on the innate ability of the young rats to survive, has not precluded the development of dental caries. The fact that the other group of nine germfree rats born normally and rat suckled has likewise shown no carious lesions provided ample evidence to substantiate this point. Second, it was interesting to observe that among the animals reared germfree on the caries-producing diet in the study, the molars which happened to develop traumatic fractures perhaps weeks before the animals were sacrificed likewise did not show evidence of caries activity. The minimal amount of stains on the teeth probably reflects the absence of chromogenic bacteria.

It is believed that the results presented in this report establish, on an unequivocal, although experimental basis, the fact that animals entirely free of living microorganisms do not develop dental caries, despite the ingestion of a diet which in conventional control rats initiates and promotes this disease of the teeth. Findings made in this base line study were not unexpected. Suggestive evidence had been accumulated earlier. For instance, it has been repeatedly shown that a consistent effort at mechanical removal of bacteria and their substrate from around the teeth as practiced in rigorous oral hygiene can minimize the incidence of dental caries. Furthermore, it has been demonstrated more recently that the use of a dentifrice containing the antibiotic, penicillin, which is growth-inhibitory to certain bacteria, can markedly reduce caries in both experimental animals and in school children. Also of similar import is the fact that there has never been reported a case of true caries in unerupted teeth, even when they have remained impacted in their germfree osseous crypts for years, while in the same individual, similar teeth exposed to a noxious environment in the oral cavity have decayed.

The major contribution of the experiments reported in this study is the demonstration, through the use of animals free of all microbes, that dental caries cannot occur in the absence of such microbes. Only in this limited sense might it be hoped that the findings herein reported may in retrospect constitute a new milestone in caries research. Since the conventional microbe-contaminated animals when fed exactly the same diet did develop the disease, it is logical to assume, although it has not been demonstrated in these particular experiments, that the disease, when it occurs, is the result of bacterial action. Thus, with the conclusive negative experimental data now made available, it must be assumed that dental caries is a disease truly caused by microorganisms.

SUMMARY

Methods and technics for adopting the procedures of germfree rearing of animals to the study of experimental dental caries have been described in this first and complete report on the initial phase of the study.

Findings indicated that twenty-two rats *(Rattus norvegicus albinus)* reared under germfree conditions remained entirely free of even microscopically demonstrable dental caries. Of thirty-nine conventional control rats, possessing the usual mixed microbial populations, thirty-eight developed carious lesions when maintained on the same kind of dietary regime as the germfree animals.

It is deduced from this evidence that dental caries in the rat is not possible in the absence of microorganisms.

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# THE EFFECTS OF A HIGH CARBOHYDRATE DIET ON THE TEETH OF PARABIOSED ALBINO RATS

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RECENT investigations[1-8] provide evidence that refined carbohydrates which are added to adequate diets fed to Wistar strain albino rats are a contributory factor in the incidence of dental caries in the molar teeth of these rats. At the same time, these studies indicate a strain difference in the cariogenicity of such sugar-containing diets. The work of Hunt and Hoppert[9] on the inheritance factor in dental caries of albino rats also demonstrated the existence of caries-susceptible and caries-resistant strains from the same stock. In view of these findings and the probable existence of other variables, such as food assimilation, sugar clearance by the saliva, tooth structure, and oral environment, it was deemed advisable to consider a new experimental approach to obtain maximum reduction in the variants between the experimental animals and the controls.

The present study is an attempt at the maximal reduction of individual variation by the use of inbred littermate albino rats in parabiosis. The growth[10] and dental responses to diet by parabiotic albino rats have led to the hypothesis that successfully parabiosed rats may be considered to live in a similar, if not equal, physiologic environment. The following evidence strongly suggests that successful parabiosis approaches the status of uniovular twins:

> (1) There are almost parallel growth increments similar to those found in un-united animals fed the same diet. (2) After three or four days of parabiotic union, there is a blood interchange every twenty-four hours between the animals, equal to at least ten times the blood volume.[11] Using $Fe^{59}$ and $Na^{24}$ isotopes, Huff, Trautman, and Van Dyke[12] calculated that 0.66 per cent of the blood volume per minute was transferred from one parabiont to the other. (3) The successful take of skin grafts is an accepted sign of genetic similarity between individuals. In successful unions, there is a complete and healthy take of the united animals' skins, with concomitant obliteration of the suture area and uniform hair growth. Subsequent successful exchange of pedicle skin grafts between parabionts suggests either homeostatic adaptation or close genetic similarity. (4) Toleration and equal regeneration of sciatic nerve trunks crossed from one parabiont to another further suggest genetic similarity. The rate of growth of the proximal sciatic stump from one parabiont into the cut distal sheath of the other parabiont is equal to that found in operated individual animals.[13]

Successful parabiosis occurs in approximately 25 per cent of all parabiotic surgery performed. The majority of unions in parabiosis are either Unsuccessful Class I or Class II. In Class I, the littermates do not grow equally, although both appear to be consuming large quantities of food. One of the parabionts remains stunted, soon appears toxic and anemic, and usually dies prior to the fifteenth day of parabiosis. Unsuccessful Class II parabiosis is characterized by

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either unequal or equal growth of the parabionts, both of whom appear to remain in good health. The suture line uniting the animals does not become obliterated, and some tearing apart of the animals occurs along this union line. The hair growth in these parabiotic pairs is not uniform, and some degree of alopecia is present in one or both.

The experimental work reported here is primarily concerned with the derivation of control data on the nature of the growth and dental responses of successfully parabiosed rats to both a balanced diet and a high carbohydrate diet containing refined dextrose.

## MATERIALS AND METHODS

*Animals.*—Wistar strain albino rats (Carworth Farms) were used. A pilot run of two groups of littermates (20 rats in each) was fed on pellets and ground balanced diet, and growth and molar dental caries were observed. Three additional groups were then formed, containing representatives from each litter when possible. *Group 1* contained twenty rats fed on the high carbohydrate diet from weaning until 120 days. *Group 2* was made up of ten pairs of parabiosed littermates fed on the ground balanced diet. *Group 3* consisted of twenty pairs of parabiosed rats, of which seven pairs met the established criteria of successful parabiosis. This group was fed on the experimental high carbohydrate diet. In all, 120 animals were studied and sacrificed at varying intervals to ascertain nutritional effects.

*Balanced Diet.*—Pellets* and finely ground food (passing through a thirty-mesh screen) of the following composition have proved adequate for normal growth, development, and reproduction. By analysis from the manufacturer, it consists of:

| | |
|---|---|
| Carbohydrate | 44.00% |
| Crude protein | 29.00% |
| Crude fat | 8.28% |
| Ash | 10.54% |
| Salt | 1.54% |

Additionally fortified with cod liver oil, milk, chlorophyll, vitamins A and B complex.

*Experimental Ration.*—Powdered dextrose, U.S.P., was added to the ground ration in equal volume and thoroughly mixed together, thus making the carbohydrate content 72 per cent instead of the 44 per cent in the previous table. This was fed to the animals ad libitum. No determination was made of the food consumed, although daily records were made of the weights of the animals.

*Environment.*—All animals were kept at a constant temperature of 75° F. with 50 per cent humidity. The animals occupied cages measuring 7 by 7 by 9½ inches having a wire screen floor and front panel. In some instances where the animals tended to rupture the skin suture area by pulling apart, a sheet metal floor was added with wood shavings.

*Parabiosis.*—A modification of the method described by Bunster and Meyer[14] was employed. Both scapulae were sutured together by passing the sutures through the central portion of the body of the bone and tying the ends together

*Big Red Dog Pellets, manufactured by Cooperative GLF Marketing Service Inc., Canandaigua, N. Y.

securely. The coelomic cavities of the parabionts were kept separate by bringing the incised ventral muscle and peritoneal flaps together and to the posterior wall flaps when suturing. Parabiosis was performed between the twenty-first and thirtieth day of life.

TABLE I

INFLUENCE OF DIET ON WEIGHT AND INCIDENCE OF DENTAL CARIES IN SINGLE AND PARABIOTIC RATS

| | SINGLE RATS | | | | PARABIOTIC RATS | | | |
|---|---|---|---|---|---|---|---|---|
| | | | DENTAL CARIES | | | COMBINED | DENTAL CARIES | |
| TYPE OF DIET | AGE (DAYS) | WEIGHT (GM.) | MANDIBLE | MAXILLA | AGE (DAYS) | WEIGHT (GM.) | MANDIBLE | MAXILLA |
| Balanced diet, pellets* | 21 | 35 | 0 | 0 | | | | |
| | 75 | 185 | 0 | 0 | | | | |
| | 100 | 275 | 0 | 0 | | | | |
| Balanced diet, ground† | 21 | 35 | 0 | 0 | 21 | 70 | 0/0 | 0/0 |
| | 75 | 150 | 0 | 0 | 75 | 270 | 0/0 | 0/0 |
| | 100 | 260 | 0 | 0 | 100 | 420 | 1/1 | 0.2/0 |
| Mixture of 50% volume of ground balanced diet and refined dextrose | 21 | 35 | 0 | 0 | 21 | 70 | 0/0 | 0/0 |
| | 75 | 135 | 1.1 | 0.1 | 75 | 260 | 1/1 | 0.4/0.4 |
| | 100 | 250 | 1.5 | 0.5 | 100 | 405 | 3/3 | 0.4/0.4 |

*Big Red Dog Pellets.
†The pellets have been ground sufficiently to pass through a thirty-mesh screen.

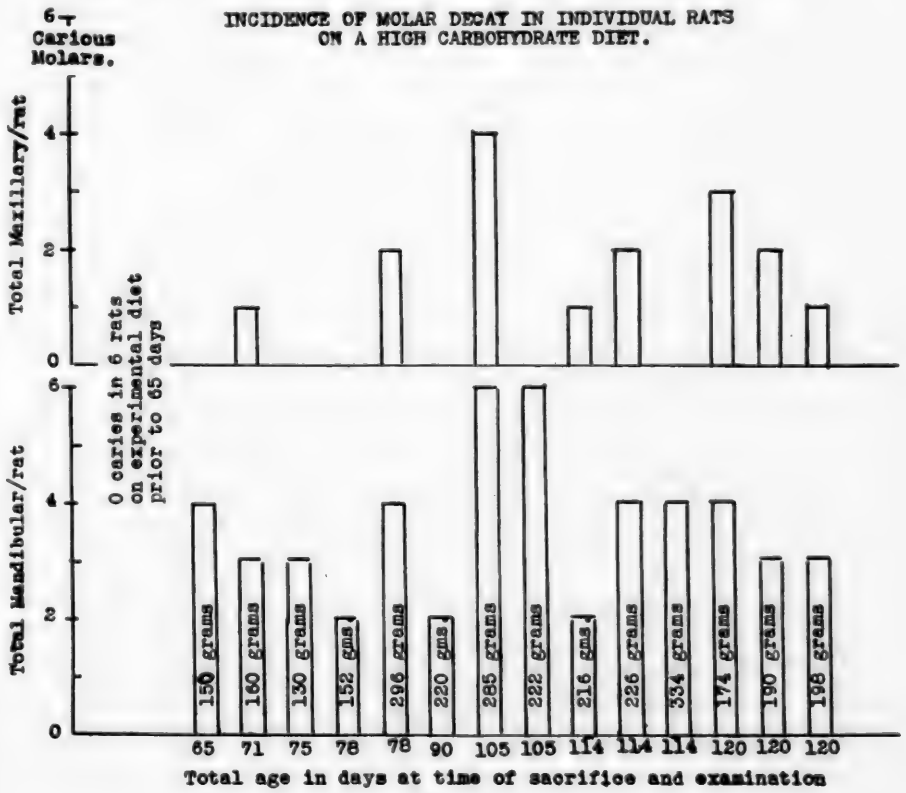


Fig. 1.

*Examination for Dental Caries.*—I was unable to ascertain, with any degree of certainty, the presence or absence of dental decay in the living rat. The procedure used was to sacrifice the animals, remove the jaws, scrub the tooth surfaces, and examine the teeth under a ×13 dissecting microscope while probing the fissures with a thin probe. The incidence of dental decay found by this method correlated very closely with the findings made by decalcification and sectioning, and by the use of aniline clearing solution suggested by Leicester and Schamp.[15] Roentgen examination by the use of dental films was unsuccessful because of the large pulp chamber and deep fissures.

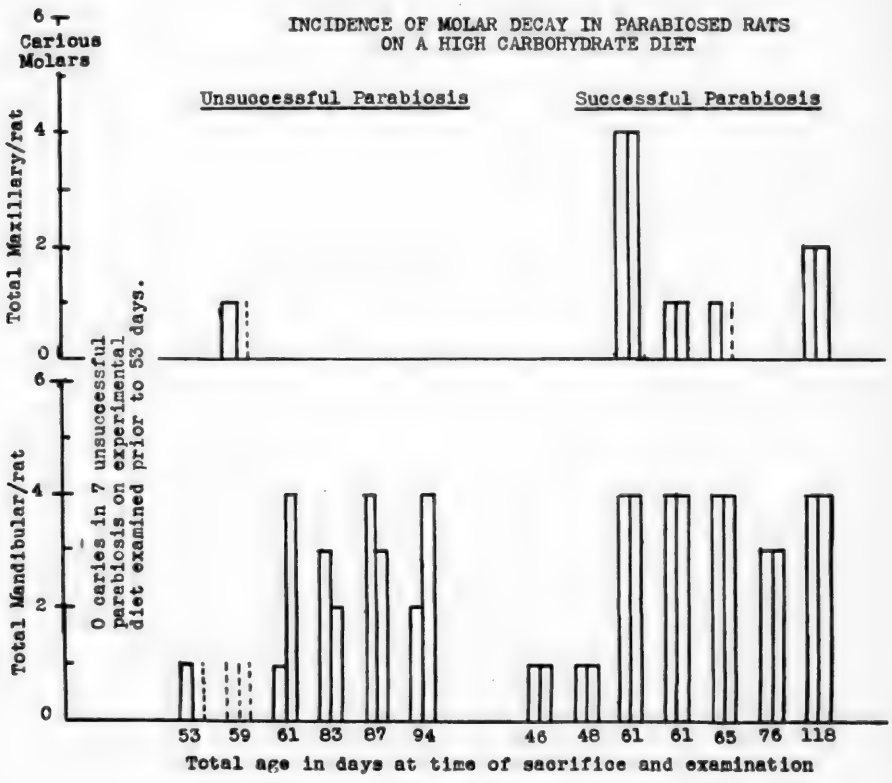
INCIDENCE OF MOLAR DECAY IN PARABIOSED RATS
ON A HIGH CARBOHYDRATE DIET
Carious Molars
Unsuccessful Parabiosis
Successful Parabiosis
Total Maxillary/rat
Total Mandibular/rat
0 caries in 7 unsuccessful parabiosis on experimental diet examined prior to 53 days.
53 59 61 83 87 94 46 48 61 61 65 76 118
Total age in days at time of sacrifice and examination

Fig. 2.

## RESULTS

The median weights for each group of animals are given in Table I. The group of rats fed on pellets was used to establish a norm, since this is the basic food for all rats used in the Department of Anatomy. Despite the small decreases in total weight gain while on the experimental diet, the animals showed no unfavorable effects or retardation of growth. Diet, in each case, started after weaning.[6]

The caries experience of the animals on the ground balanced diet and on the high carbohydrate diet (dextrose, U.S.P. mixture) is indicated in Table I and Figs. 1 and 2. From these data the following interpretation is made: (1) The addition of refined dextrose to a balanced noncarious-producing diet increased the cariogenicity of the diet to a considerable degree. (2) Parabiosed animals are apparently more susceptible to the caries-producing properties of refined carbohydrate. (3) Although, while on the cariogenic diet, there appears to be an increase in the incidence of mandibular molar caries from the seventy-fifth day to the one hundredth day, no significant correlation was found. (4) Only fissure type caries were found, and no fracturing of the enamel was observed. (5) Of significance in future dental research was the finding that successfully parabiosed Wistar strain rats demonstrated such genetic similarity that the pattern and incidence of dental decay were almost identical in both parabionts.

## DISCUSSION

By the addition of 50 per cent by volume of dextrose to a balanced ground diet it was found possible to change an apparently caries-resistant strain of rats to caries susceptibility. This finding is similar to that of Shibata[1] and Sognnaes.[16]

The rapidity of caries formation on this experimental diet is also noteworthy. Keller, Hunt, and Hoppert,[17] reporting on a caries-susceptible albino rat strain, found that mandibular molar caries was induced 28.6 days and maxillary molar caries 315.6 days after weaning. In the series reported here, an apparently caries-resistant strain of albino rats developed dental caries (demonstrable fissure caries in upper and lower molars between the thirtieth and fortieth day after weaning) when refined carbohydrate (dextrose) was added to the diet. Although the exact mechanism of action of this refined carbohydrate fraction in the formation of dental caries is still uncertain, it fits in well with the presently held hypothesis of carbohydrate degradation and concomitant decalcifying pH values. The use of the monosaccharide dextrose should facilitate such degradation and theoretically promote the early appearance of fissure caries.

The utility of parabiosis as a dental caries research tool is illustrated in Fig. 2. In a group of seven pairs of albino rats in successful parabiosis, fed the high carbohydrate diet, the incidence of mandibular molar decay was found to be identical in both parabionts. Only in the case of one pair of successful parabionts was there some question of identical maxillary molar caries. This small series strongly suggests the reduction of individual variation between the two animals.

## SUMMARY AND CONCLUSION

1. A strain of albino rats resistant to dental caries on an adequate balanced diet developed caries on the addition of 50 per cent by volume of refined dextrose (high carbohydrate) to the balanced diet.

2. Successfully parabiosed albino rats on such a high carbohydrate diet will develop equal and similarly patterned mandibular molar caries activity.

3. Successful parabiosis of animals suggests a method whereby the numerous individual variations which thwart the solution of the etiology of dental caries may be overcome.

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# A METHOD OF CULTIVATING TOOTH GERMS IN CARREL FLASKS

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IT IS generally accepted that the best results of organized growth in vitro have been achieved when the tissue is cultured in a medium of limited growth-stimulating properties with a minimum of disturbance to the explant.[1] An investigation of a simple method for the in vitro cultivation of tooth germs, a problem in organized growth, should start with these known requirements. Furthermore, it is desirable to observe and adjust the pH, and to study and photograph the cultures in the living state.

Investigations of the in vitro cultivation of tooth germs to date have utilized the hanging drop, watch crystal method, and the depression slide.[2-8] The Carrel flask (Fig. 4) was chosen as the vehicle for the cultivation of tooth germs because it is so admirably suited to the conditions of organized growth. The medium may be varied and the tissues may be kept in the same flask for long periods of time.[9] Waste products of cell metabolism may be removed by washing, and fresh nutrient fluid added without transferring the explant. Alterations in pH are readily observed and maintained with facility. Cultures grown in Carrel flasks may easily be examined and photographed during the course of the experiment.

The Carrel flask method is adequately reported in standard textbooks.[10, 11] Whenever the technic followed that of a prescribed text, references will be given. Procedures developed during the investigation will be described in detail.

*Preparation of Glassware.*—All glassware should be of the best quality, such as Pyrex, and must be chemically clean. For washing, a detergent was used instead of soap. Demineralized water was substituted for distilled water because the low organic content of the tap water made this convenient method of removing electrolytes practical. The alcohol rinse before drying was omitted.[12, 13]

The flasks and pipettes were washed separately. The flasks were rinsed with cold tap water, then boiled not less than twenty minutes in the detergent, care being taken to fill each flask with the detergent solution before placing it in the receptacle for boiling. Each was rinsed with hot running tap water, as recommended by Cameron,[14] and after three rinses with demineralized water, it was placed in an oven at 80° C. until dry. Pipettes were boiled in the detergent, which was then removed in an automatic pipette washer.[15] To insure a thorough rinsing, demineralized water was pulled through the pipettes using suction.

The other glassware, such as tubes, glass plates, hollow ground slides, Petri dishes, and watch crystals, were rinsed with cold tap water, and then boiled at

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least twenty minutes in the detergent. The detergent was removed with hot running tap water, and the glassware rinsed three times with demineralized water and dried in the oven at 80° C.

Instruments were rinsed with cold running tap water and then cleaned with the detergent. They were rinsed with hot running tap water and demineralized water, dried immediately with a clean sponge, and placed in the oven.

New rubber nipples for the pipettes and rubber stoppers were first boiled with sodium bicarbonate and scrubbed clean.[16] They were then boiled in the detergent and thoroughly rinsed with tap water and demineralized water. New rubber tubing was first boiled with sodium bicarbonate, and then boiled in the detergent. It was thoroughly rinsed with hot running tap water and demineralized water.

Supplies were wrapped and sterilized according to standard procedure.[17]

*Culture Room.*—Rigid observance of the rules of asepsis should never be relaxed. The pouring of Tyrode solution, reconstituting of lyophilized chicken plasma and chick embryo extract, preparation of rat embryo extract, preparation of standard pH flasks, dissection of the tooth germs, and all culture procedures were carried out in a relatively dust-free room.[18] Masks, surgical caps, and gowns were worn while working sterilely. Since tissues are better observed against a dark background, black table covers were used (Fig. 1). The openings of tubes and flasks were always flamed before using. Sterile rubber stoppers were also flamed as an added precaution before inserting in a tube or flask. The pipettes, after the nipples were in place, were passed through the flame before inserting in the protective tube in the rack (Fig. 1). Any material suspected of contamination due to faulty technic was discarded.

*Preparation of Tyrode Solution.*—The isotonic salt solution used in all experiments was Tyrode.[19] Concentrated stock solutions were prepared by weighing on a triple-beam balance to the nearest 10 mg. the following salts:

| | |
|---|---|
| Sodium chloride (NaCl) | 16.00 Gm. |
| Potassium chloride (KCl) | 4.00 Gm. |
| Calcium chloride ($CaCl_2$) | 4.00 Gm. |
| Magnesium chloride ($MgCl_2 \cdot H_2O$) | 2.00 Gm. |
| Sodium phosphate monobasic ($Na_2HPO_4 \cdot H_2O$) | 1.00 Gm. |

Each of these was dissolved in pyrogen-free distilled water and diluted to 100 ml. in volumetric flasks. The solutions were sealed with parafilm and stored at 3° C.

One liter of Tyrode solution was prepared as follows: Stock sodium chloride, 50 ml.; stock potassium chloride, 5 ml.; stock calcium chloride, 5 ml.; stock magnesium chloride, 5 ml.; and stock sodium phosphate, 5 ml., were placed in a one-liter volumetric flask with about 500 ml. of pyrogen-free distilled water. One gram each of sodium bicarbonate ($NaHCO_3$) and glucose, freshly weighed, were added to the solution and the mixture made up to volume. The sodium bicarbonate, because of the loss of carbon dioxide on standing, was not prepared as a stock solution, nor was the glucose, because it is a good medium for bacterial growth.

All the equipment for filtering and pouring the Tyrode solution was wrapped for autoclaving in one package and assembled when the solution was

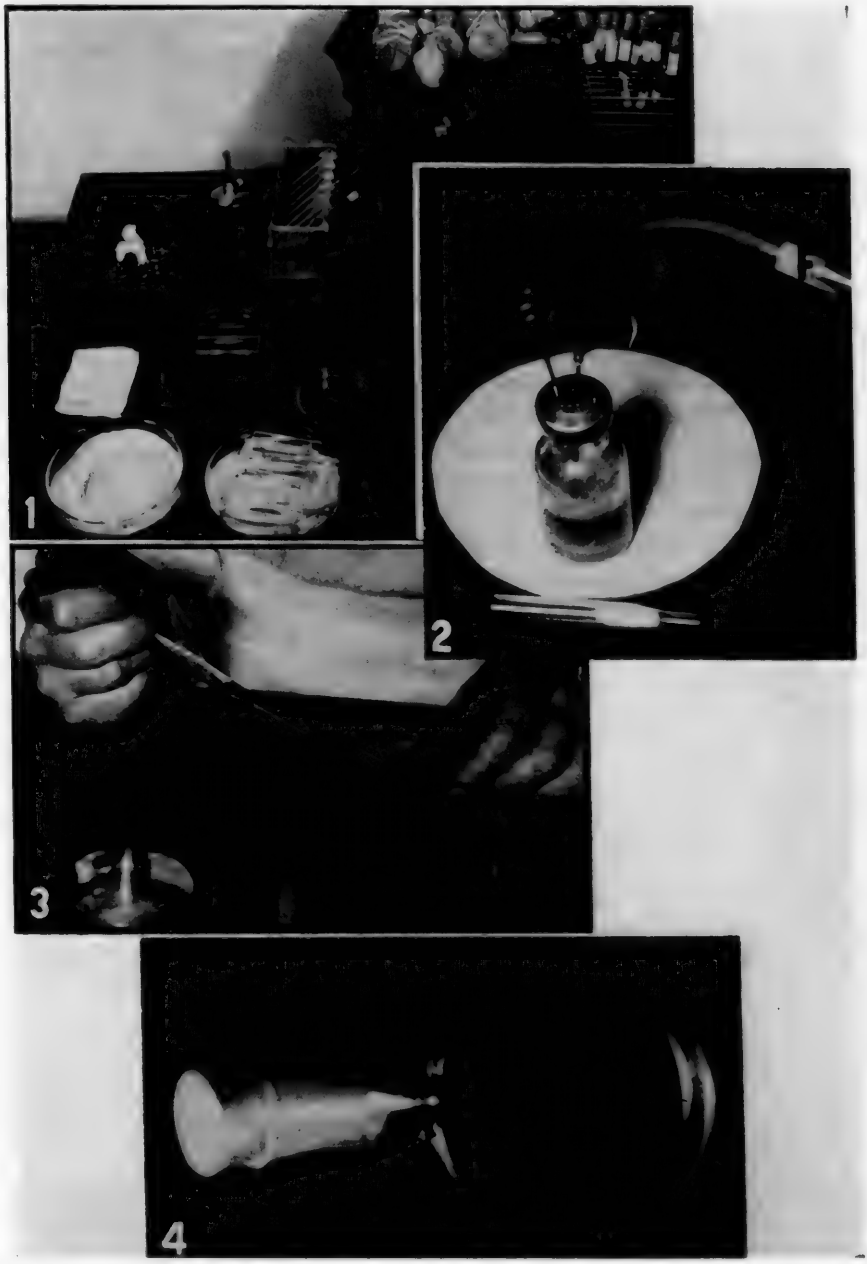


Fig. 1.—Culture table showing hollow ground slides, watch crystals, sterile sponges, Carrel flasks, microburner, rack with pipettes and instruments, forceps and platinum spatula in protective tubes, Petri dish, accessory table with extra sterile glassware, and media.

Fig. 2.—Acidifying lyophilized plasma. Carbon dioxide passes through filter (*F*), sterile tubing, and hypodermic needle into reconstituted plasma. Gas escapes through second hypodermic needle. Note construction of filter.

Fig. 3.—Adding or withdrawing media with pipette.

Fig. 4.—Carrel D 3.5 flask with two tooth germs ready for incubation.

ready to be sterilized.[20] The Seitz filter was the method chosen for sterilization, using suction to pull the solution through the filter. Cameron[21] recommended sterilization by autoclaving.

*Indicator for Hydrogen-Ion Concentration.*—The indicator used for the observation of pH was phenol red. Since a concentration of 0.005 per cent is not toxic to tissues,[22] it was added to all media used in the flasks. The indicator was obtained commercially at a concentration of 0.5 per cent from which further dilutions were made.

*Preparation of pH Standards.*—Phosphate buffer solutions, M/15, for the determination of pH were prepared as follows: (1) 9.47 Gm. of dibasic sodium phosphate ($Na_2HPO_4$) "Sørensen purity" was dissolved in pyrogen-free distilled water in a volumetric flask and diluted to 1,000 ml.; (2) 9.08 Gm. of potassium acid phosphate ($KH_2PO_4$) "Sørensen purity" was dissolved in pyrogen-free distilled water in a volumetric flask and diluted to 1,000 ml.

The desired pH ranges were obtained by mixing appropriate volumes of these two solutions.[23] A sufficient quantity of 0.5 per cent phenol red was added to achieve a final concentration of 0.005 per cent in the buffers. These solutions were sterilized by autoclaving, and were stored in the refrigerator at 3° C. The sterile buffered salt solutions were used to prepare the standards for pH observation in the experimental flasks. The volume of the buffer solution corresponded to the total volume of media used in the culture flasks, and was prepared sterilely in the same size flask as that used in the experiment. The sterile standard flasks were tightly stoppered with sterile rubber stoppers and sealed with paraffin. These flasks maintained their pH range for months.

*Preparation of Plasma.*—It is generally agreed that cells in vitro need a semisolid support for continued growth and activity. Chicken plasma has been found to be satisfactory as a coagulum for cells of many species.[24] Any factor in the avian plasma that might be toxic to mammalian tissues is present in small quantities and is not stable.[25]

Chicken plasma may be obtained in several ways.[26, 27] Comparison of cultures in plasma obtained by cardiac puncture and lyophilized plasma showed no difference.[28] Lyophilized plasma was prepared by first adding 0.005 per cent phenol red to the reconstituting fluid and then bringing the plasma to volume. To accelerate coagulation, the reconstituted plasma was acidified by passing carbon dioxide gas into the solution until the phenol red indicator changed in color from red to yellow (Fig. 2).

*Preparation of Extracts.*—Lyophilized chicken embryo extract was brought to volume with the same reconstituting fluid used for the chicken plasma. The rat embryo extract was prepared from the bodies of the embryos after the heads had been excised for the dissection of the tooth germs. The bodies were placed in a watch crystal and washed with Tyrode solution. The tissue was chopped to a fine pulp with a curved scissors, transferred to tubes with a coarse tipped pipette, and centrifuged for twenty minutes at 2,000 r.p.m. The supernatant

fluid was removed and diluted with Tyrode solution to a 25 per cent concentration. This was considered 100 per cent rat embryo extract, from which further dilutions were made. All dilutions of extract contained 0.005 per cent phenol red.

*Preparation of Flasks.*—To form the clot, 1.5 ml. of plasma was introduced into the flask to which was added 0.5 ml. of nutrient fluid. The media were thoroughly mixed in the flask. The tooth germs were planted in the clot before coagulation. Tissue may be placed on the clot as well in a Carrel flask.

After coagulation, the surface of the substrate was wetted with Tyrode solution and then withdrawn (Fig. 3). This facilitated spreading of the supernatant fluid. Nutrient fluid, 0.5 ml., diluted with Tyrode solution to the same concentration as that used in the clot was then added to the flask. The pH was adjusted, the flask closed with a sterile rubber stopper, sealed with paraffin, and incubated (Fig. 4).

*pH Adjustment.*—The pH may be adjusted by filling the flask with a gas mixture of oxygen, carbon dioxide, and nitrogen.[29] However, if the gas mixtures are not available, a simple method for reducing pH is to blow exhaled air into the flask through a sterile pipette, to which is attached a sterile cotton filter and sterile rubber tubing (Fig. 5), until the desired pH is obtained. If the pH is low, the flask is left unstoppered, the opening protected with a sterile sponge, until sufficient carbon dioxide has diffused to the outer air to raise the pH to the desired range (Fig. 6).

*Washing and Renewing Nutrient Fluid.*—Since the waste products of cell metabolism may be washed out of the clot and new nutrient fluid added, the need for transfer is greatly reduced. Rat tooth germs were washed and fresh nutrient added at either two- or four-day intervals.[28] The supernatant fluid was withdrawn with a pipette (Fig. 4), and the clot washed for thirty minutes by adding Tyrode solution containing 0.005 per cent phenol red. The flasks were closed with sterile rubber stoppers and placed in the incubator while being washed. After washing, the Tyrode solution was removed with a pipette (Fig. 4), and 0.5 ml. of the nutrient fluid was added. The pH was adjusted, and the flasks sealed and incubated. None of the cultures was transferred. The maximum period of cultivation was twenty days.

*Photography.*—Photography provides the only efficient means of recording the appearance of living tooth germs. Tooth germs in Carrel flasks may be conveniently photographed with the ordinary microscope if the flask is inverted. The oblique neck necessitates a special stand which will provide a flat working surface. A suitable stand was constructed of a cylinder of dental wax of the same diameter as the flask. A recess accommodated the neck of the flask. The cylinder was attached by heat to a glass slide, making it possible to use the mechanical stage for observation and photography (Fig. 7).

*Preparation of Tooth Germs for Fixation.*—When the tissues were ready for fixation, the entire coagulum was removed from the flask[30] and transferred to a glass plate. The tooth germs were cut out of the clot and the outgrowth

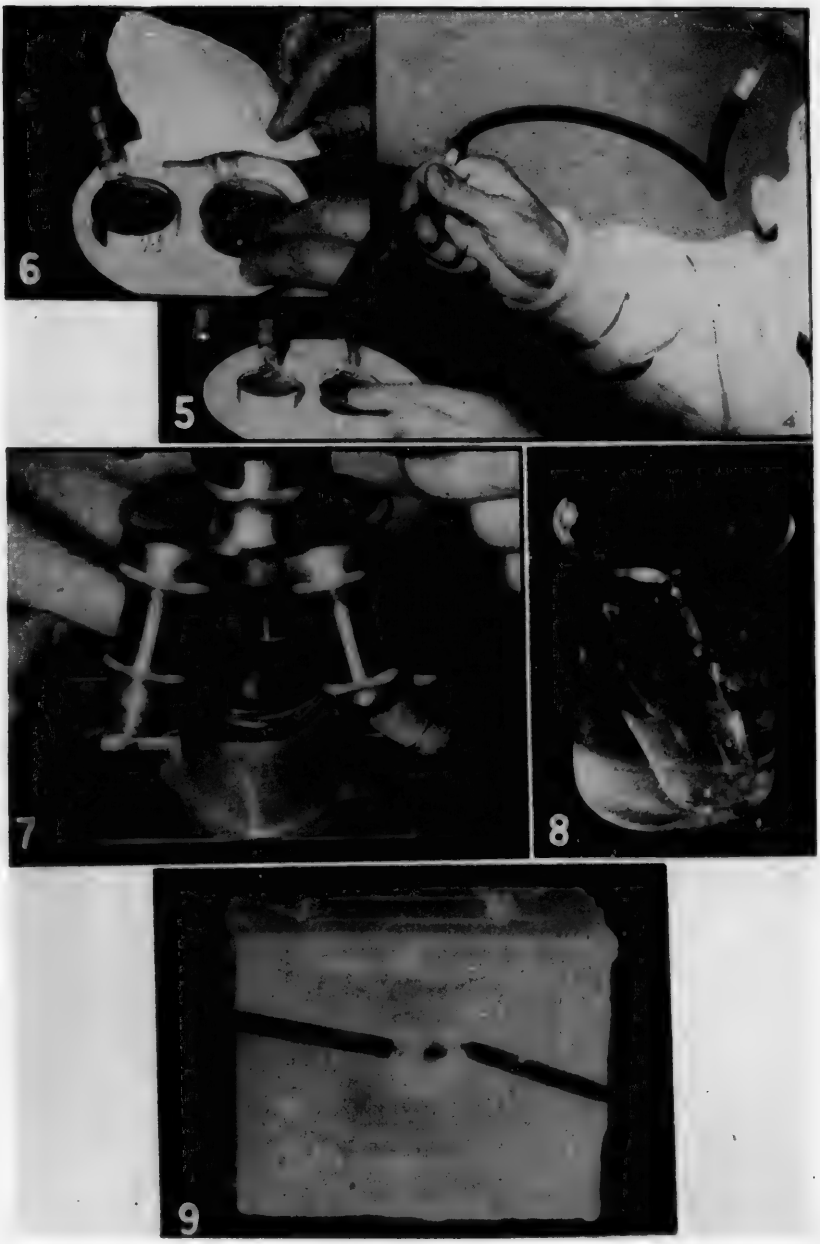


Fig. 5.—Reducing pH by blowing exhaled air into flask. Note pH standard.

Fig. 6.—Raising pH by exposing flask to air. Note pH standard.

Fig. 7.—Method of examining and photographing living tooth germs.

Fig. 8.—Perforated glass tube in beaker for histologic technique. Beaker covered with parafilm to prevent evaporation.

Fig. 9.—Tissuemat block with recess. Tooth germ oriented with dissecting needles.

was dissected away under the dissecting microscope with two Wheeler discission knives. It was desirable to remove the outgrowth from the tooth germ to facilitate orientation for sectioning.

*Histology.*—The histologic technic presented two major problems. Tooth germs treated by the conventional method were lost because of their size. After fixation the tissue shrinks and cannot be easily seen, particularly after clearing.

In order to treat a larger mass of tissue, an attempt was made to orient the explant in a clot. A recess cut in Gelfoam was filled with plasma in which the tooth germ was oriented for sectioning. Coagulation was hastened by adding thrombin. The Gelfoam containing the clot and the tooth germ was treated as a large piece of tissue. The results were unsatisfactory. Excessive shrinkage occurred and the orientation of the tooth germ, which apparently changed position before coagulation of the clot, was bad.

A more satisfactory method consisted of orienting the tissue after paraffin infiltration. A small tube with minute perforations at the base was constructed. It was placed in a beaker (Fig. 8) which was covered with parafilm to prevent evaporation of solutions. After removal from the clot, the tooth germ was transferred to the perforated tube in the beaker containing fixative. The solutions were pipetted off and fresh solutions added without disturbing the tissue. During dehydration the tooth germ was stained with alcoholic eosin to make it more visible. At the benzine paraffin stage, the tissue was transferred to a small Stender jar and infiltrated with tissuemat. A depression roughly 5 by 10 mm. was cut into a rectangular block of tissuemat having a melting point of 54 to 56° C. The tooth germ was oriented for axial sections in the depression using two warm dissecting needles (Fig. 9). The depression was then filled with 60 to 62° tissuemat, and when a surface film formed, the block was dropped into ice water. Tooth germs treated in this fashion did not shrink more than normally, and the orientation was good.

Difficulties were encountered when the blocks were cut. Air bubbles were occasionally entrapped near the tissue. Unless the two tissuemats fused completely, the block fell apart and the tissues had to be re-embedded. Further investigation on this aspect of the problem is in progress.

## SUMMARY

The Carrel flask technic is a suitable method for the in vitro cultivation of tooth germs.

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# CULTIVATION OF RAT MOLAR TOOTH GERMS IN CARREL FLASKS. I

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THE in vitro culture of tissue is an accepted experimental method which may be utilized to elucidate problems of growth, differentiation, organizer influence, and function. It has also contributed to studies of malignant cells and the effect of external influences on both normal and pathologic tissue.

Tissue culture is concerned with two aspects of growth. Unorganized growth, culturing of a single type of cell, is concerned with survival and proliferation. Organized growth deals with the differentiation of growing tissues and the development of their functional capabilities.[1] The in vitro culture of tooth germs is a problem in organized growth.

The results of previous investigations of tooth germ culture are both meager and gratifying. The findings verified some existing concepts of dental embryology and made further contributions to the field. Pinkerton and Boyle[2] cultured the developing teeth of newborn kittens and observed that the ectoderm and mesoderm grew freely and tended to differentiate. The major contribution to the field was made by Glasstone.[3-5] She cultured tooth germs of embryos, which provided sterile tissue from which to dissect the explants. She reported that tooth germs displayed remarkable powers of development. Cultivating rat and rabbit tooth germs, she reported the presence of odontoblasts and dentin formation. In rabbit cultures some enamel had formed. Nuckolls[6] and Losee[7] cultivated mouse and rat tooth germs. They used the Long and Evans technic to determine the age of the rat embryos. Nuckolls reported a relationship between the enamel organ and the bony crypt. Losee cultured one tooth germ for seventy-eight days and reported that the tissue was viable before fixation. Hay[8] is presently culturing tooth germs of mice, and corroborates previous findings. She is also culturing areas of the dental lamina at early stages, and reports development of an incisor with dentin formation on the labial surface. Szabo[9] is investigating the growth potency of the cells of the tooth germ by culturing undissected mouse molar tooth germs or the extirpated formative elements of the incisors. He compared fluid media with the hanging drop method and found evidence of differentiation in the enamel epithelium.

A preparatory investigation determined the daily progress of growth and development, which established a table of standards for the stages at which the in vitro culture was started. Results of experiments were similarly measured. References to the stage of development and the terminology are those established in the report on the odontogenesis of the rat molar.[10]

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The object of this study is to determine the applicability of the Carrel flask technic to the in vitro culture of tooth germs and to explore its methodology. Two aspects of these possibilities were studied in an effort to establish a standard technic. This report is concerned with "in the clot" cultivation of undissected and dissected tooth germs of known age.[10] A subsequent investigation of "on the clot" cultivation is in progress and will be reported at a future time.

The first experiments served to compare lyophilized chicken plasma with that obtained by cardiac puncture using heparin as an anticoagulant. No appreciable difference was noted. In the majority of the reported studies, the clot was made of lyophilized chicken plasma with either chick or rat embryo extract.[11] In each study, only the body of the rat embryo was used to produce extract. Thus the age of the rat embryo extract was the same as the age of the explant throughout its in vitro culture. All nutrient media and plasma contained 0.005 per cent phenol red as an indicator. The explant was kept in the original clot, washed, and supernatant fluid added at regular intervals.

The results show that the viability of the cells of tooth germs is better maintained with rat embryo extract than with chick embryo extract. Undissected tooth germs degenerated although the enamel epithelium showed a greater potency for survival than the fibroblasts of the dental papilla. Dissected tooth germs cultured in the clot exhibited approximately two days of development regardless of the period of cultivation.

### CULTIVATION OF UNDISSECTED TOOTH GERMS

In order to eliminate trauma and operative injury to the tooth germ, an attempt was made to cultivate entire mandibles or sections of the mandible and maxilla containing tooth germs. Forty such explants containing undissected molar tooth germs were cultivated, ranging in embryonic age from 18 to 21 days. The same investigation served to compare the results of cultivation in rat and chick embryo extract.

Comparison of lyophilized chick embryo extract and rat embryo extract was also made in concentrations of 50, 25, and 12½ per cent of the medium. The supernatant fluid diluted in Tyrode solution was of the same concentration as that in the clot. The pH was maintained at 7.6. Tissues were washed with Tyrode solution, and fresh nutrient media added every two days. The explants were not transferred. The average period of cultivation was eight days.

In all cases, the undissected tooth germs showed varying degrees of degeneration. The peripheral tissues survived and proliferated as indicated by outgrowth. The explant represented a relatively large mass of tissue, and it appeared obvious that the nutrient media did not permeat it (Figs. 1 and 2). A similar observation was reported by Glasstone.[3] The most extreme cases showed complete degeneration of the tooth germs which lay deepest in the explant. Concentration of embryo extract had no apparent effect on the result. In all, neither growth nor development was observed.

One fruitful, although not unexpected observation, was that less degeneration occurred with rat embryo extract than with chick embryo extract. Fig.

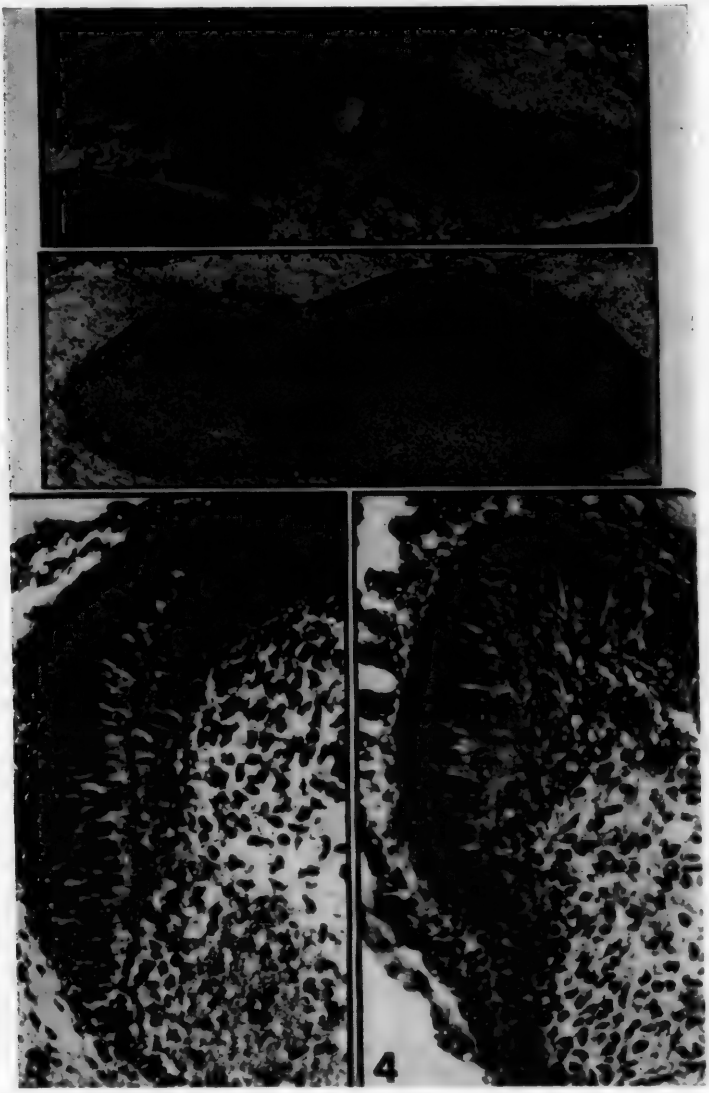

Fig. 1.—Undissected 21-day tooth germ cultivated six days in clot of 75 per cent chicken plasma and 25 per cent chick embryo extract. Supernatant fluid was 25 per cent chick embryo extract in Tyrode solution. Note dedifferentiation of enamel epithelium. *D*, Area of degeneration. Original magnification ×82, reduced ⅔.

Fig. 2.—Undissected 21-day tooth germ cultivated six days in clot of 75 per cent chicken plasma and 25 per cent rat embryo extract. Supernatant fluid was 25 per cent rat embryo extract in Tyrode solution. Note dedifferentiation of enamel epithelium. *D*, Area of degeneration. Original magnification × 82, reduced ⅔.

Fig. 3.—Area of cultivated dissected 21-day tooth germ. Media and period of cultivation same as in Fig. 1. Note predentin formation, differentiation of odontoblasts, area of degeneration, *D*. Original magnification ×600, reduced ⅔.

Fig. 4.—Area of cultivated dissected 21-day tooth germ. Media and period of cultivation same as in Fig. 2. Note formation of predentin, differentiation of odontoblasts, mitosis of fibroblasts, and preameloblasts. Note degeneration occurred. Original magnification ×600, reduced ⅔

1 shows the result of six days' cultivation of a section of the mandible of a 21-day embryo containing the tooth germs. The media consisted of 75 per cent chicken plasma and 25 per cent lyophilized chick embryo extract to form the clot. The supernatant fluid was 25 per cent chick embryo extract in Tyrode solution. There is degeneration in the center of the explant which involves the papillary area of the tooth germs. The epithelium outlines the periphery of the enamel organ but cannot be identified as such. It dedifferentiated into stratified squamous epithelium with some cuboidal cells on its inner surface. The papilla has almost completely degenerated. The more peripheral tissues are viable.

Collateral investigations were made of similar explants in which rat embryo extract replaced the chick embryo extract in the same concentration. All other factors were unchanged. Fig. 2 is a photomicrograph illustrating the result. Slight degeneration of the cells of the papilla has occurred. The epithelium has dedifferentiated into squamous epithelium in some areas, reducing to a single layer of cells.

The age of the explant within the range studied made no significant difference. Cultivation for longer periods of time resulted in complete degeneration of the tooth germs with both media.

### CULTIVATION OF DISSECTED TOOTH GERMS

Dissected tooth germs were also studied for development, growth, and survival in media containing either chick or rat embryo extract. The clot was made of 75 per cent chicken plasma with 25 per cent chick or rat embryo extract. The supernatant fluid contained the same concentration of extract in Tyrode solution.

Thirty dissected tooth germs from 20- to 21-day embryos were cultivated. The explant was washed in Tyrode solution after four days, and supernatant fluid added. The period of cultivation was six days.

Tooth germs from 21-day embryos cultivated in chicken plasma and chick embryo extract showed degeneration of the fibroblasts in the deeper area of the explant. The remaining fibroblasts and the enamel epithelium were viable after six days of cultivation. Development of one to two days occurred (Fig. 3), and was manifested by the formation of a layer of predentin and beginning differentiation of odontoblasts.

In contrast, the substitution of rat embryo extract for chick showed the same development with no degeneration. Fig. 4 illustrates a typical result of the in vitro culture of a 21-day embryo tooth germ. No degeneration occurred. Predentin formed, and differentiation of the odontoblasts established about two days' development. Cell division of preameloblasts and fibroblasts provided evidence of viability and growth. It further demonstrated that survival was better with rat embryo extract.

### IN VITRO CULTURE OF DISSECTED TOOTH GERMS IN RAT EMBRYO EXTRACT

Having established that tooth germs survived better in media containing rat embryo extract than in that containing chick, explants of various ages were studied. In some cases, the time of cultivation was increased. Seventy-three dis-

sected tooth germs were cultivated in 75 per cent chicken plasma and 25 per cent rat embryo extract. The supernatant fluid consisted of 25 per cent rat embryo extract in Tyrode solution. The periods of cultivation ranged from two to twenty days. Cultures of 20- and 21-day embryo tooth germs were fixed at two-day intervals. Seventeen-, eighteen-, and nineteen-day embryo tooth germs were fixed at four-day intervals. All cultures were washed at four-day intervals, and fresh nutrient media added. The pH was maintained at 7.6.

Dissection of 21-day embryo tooth germs proved difficult.[12] The results of in vitro cultivation at this age are inconclusive because of the small number of explants. Furthermore, the periods of cultivation were short. Nevertheless, the results are fairly consistent with observations of development made on cultures of 18-, 19-, and 20-day embryos.

Regardless of the age of the explant or the length of time of culture, the amount of development measured by in vivo standards[10] was approximately two days, and this occurred in the first four to six days of cultivation. The cells remained viable up to the time limit of the experiment. There was no degeneration.

Fig. 4 shows the development of a 21-day embryo tooth germ after six days' cultivation. It is taken from the series used to compare chick and rat embryo extracts. One to two days' development occurred in four days of cultivation.

Tooth germs from 20-day embryos were cultivated from two to six days. The maximum development of two days was achieved at four days in vitro although the cells remained vital up to the time of fixation at six days. Fig. 5 shows a dissected tooth germ after six days' cultivation. The cells are all viable, there being no evidence of degeneration. Cusp outline has advanced. The outer tunic has been invaded by fibroblasts, and young fibrous tissue is present in the central cell area. Under higher magnification (Fig. 6) the state of development may be more readily recognized. Predentin formation and the first expression of histodifferentiation of odontoblasts may be seen. Cell division is progressing in the preameloblast layer. There has been approximately two days of development. Another 20-day tooth germ, similarly cultivated for four days, exhibited even more growth and development. Fig. 7 shows that odontoblast formation has occurred, and a layer of tubular predentin is present. Preameloblasts have differentiated, showing terminal bars and Tomes's processes. In other areas, cell division of the preameloblasts is still evident. The inner cells of the stratum intermedium are differentiating. The section is equivalent to that seen at one day after birth and represents three days' growth and development in vitro.

Tooth germs from 19-day embryos were cultivated from four to twenty days. In some cases the follicle was dissected away and in others it was retained. The cells of the explants remained viable until fixation. After the termination of each period of cultivation it was noted that when the follicle was removed, the tooth germ appeared to be like a concave disc. The maximum development was achieved after eight days of cultivation. The same changes were observed in twenty-day cultures as in eight. The cells remained viable and mitosis continued although only approximately two days of development ensued.

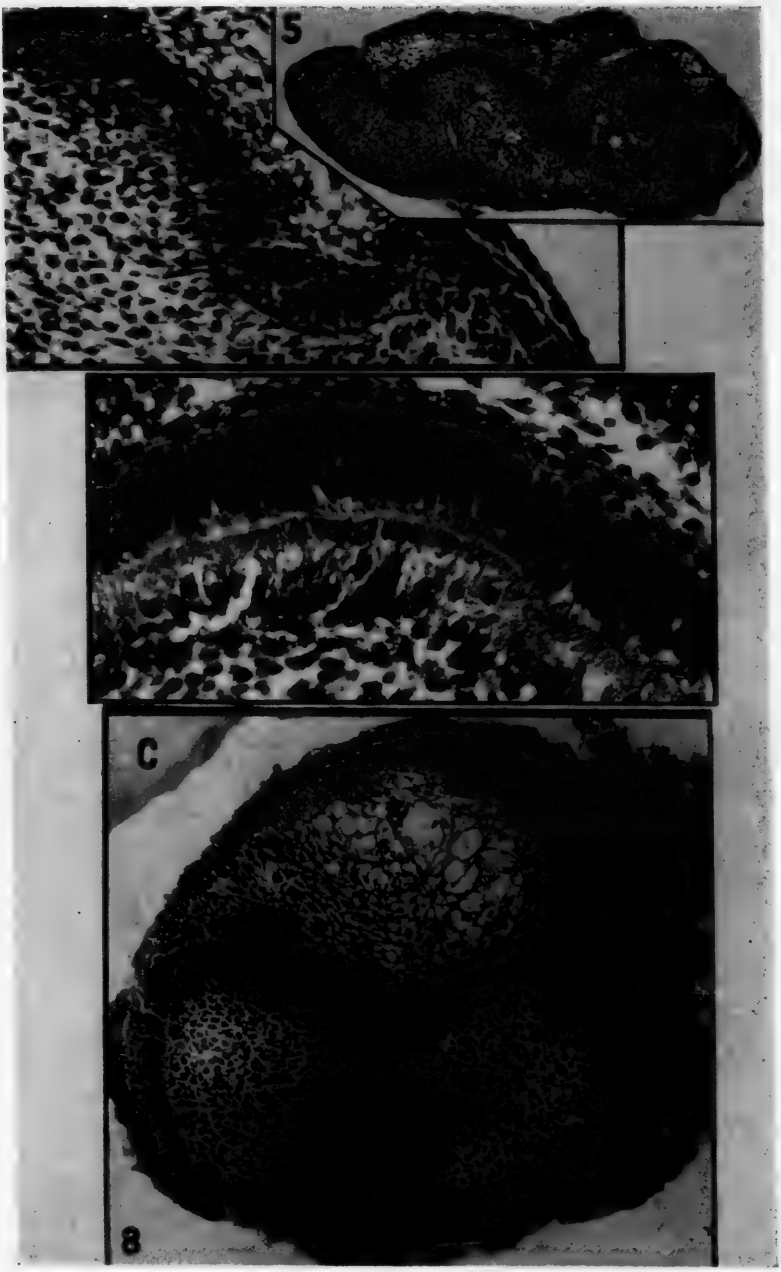


Fig. 5.—Cultivated 20-day tooth germ dissected with follicular wall. Media and period of cultivation same as in Fig. 2. Note advance in cusp outline, fibroblast invasion of central cell area. No degeneration. Section cut mesiodistally. Original magnification ×130, reduced ⅔.

Fig. 6.—Higher magnification of circumscribed area in Fig. 5. Note predentin formation, beginning of differentiation of odontoblasts. Original magnification ×600, reduced ⅔.

Fig. 7.—Area of dissected 20-day tooth germ cultivated in the same media as in Fig. 2 for four days. Note differentiation of odontoblasts, formation of tubular dentin. Arrow points to differentiated ameloblasts showing terminal bars and Tomes's processes. Inner layer of stratum intermedium differentiating into cuboidal epithelium. Original magnification ×920, reduced ⅔.

Fig. 8.—Cultivated 19-day tooth germ dissected with follicular wall. Media same as in Fig. 2. Period of cultivation eight days. Note advance in cusp outline, invagination of proliferative zones, fibrous invasion of central cell area, formation of predentin. *C*, Clot. *O*, Outgrowth. Section cut buccolingually. Original magnification ×300, reduced ⅔.

Fig. 8 is a photomicrograph of a 19-day embryo tooth germ which had been cultivated for eight days. The follicle was not excised and the explant retained its normal morphology. Cusp outline has advanced, indicating cell division of the preameloblasts. There is evidence of growth by invagination of the proliferative zone. Connective tissue invasion of the central cells has occurred. Mitosis of the preameloblasts and predentin formation may be seen. After twelve days of cultivation (Fig. 9) the same development is evident. The section illustrates the flattened appearance of a cultivated tooth germ which had had the follicular wall removed. Cultivation for twenty days (Fig. 10) resulted again in the same development. Cusp outline appears irregular, which may be an abnormality of cultivation. Oblique sectioning complicates the interpretation. Connective tissue in the central cell area is similar to that seen at eight days. Under higher magnification (Fig. 11), cell division in the proliferative zone indicates growth. No degeneration has occurred. All the cells are viable and continue to divide although there had been but two days of development.

Eighteen-day embryo tooth germs were cultured up to sixteen days. Fig. 12 illustrates the progress after eight days of cultivation. The section is cut buccolingually, and demonstrates advanced cusp outline. The presence of fibroblasts in the central cell area of the enamel organ, the fusion of outer tunic and middle layer, and the presence of a basement membrane underlying the preameloblasts establishes two days of development. An epithelial pearl may be seen over the outer tunic. After sixteen days' cultivation, the cells of the tooth germ are viable (Fig. 13). The section, cut obliquely, demonstrates essentially the same development observed in eight days. The basement membrane is slightly more advanced. Mitosis of preameloblasts is evidence of growth and cusp formation similar to that shown in Fig. 12.

Dissections of 17-day tooth germs were most difficult, and the results inconclusive.

### OBSERVATION OF LIVING TOOTH GERMS

Living tooth germs may be observed with the Carrel flask technic. The flask is placed on a stand and observed at low magnification.[11] The thickness of the explant makes high magnifications impossible.

When first planted, the tooth germ appears as an opaque oval body. Unfortunately, it is impossible to orient a tooth germ when planting, so that the surface observed may or may not show development. After photographing the explant at different intervals, it seemed evident that growth occurred.

Fig. 14 represents four different stages in vitro culture of the 19-day embryo tooth germ seen in Fig. 10. One day after planting (Fig. 14, *A*), the tooth germ appeared as a round mass of tissue. On the eighth day (Fig. 14, *B*), it assumed an oval shape and became somewhat translucent. The preameloblasts were readily discernible, and it was established that the position of the explant permitted observation of the mesial or distal surface. There was a definite increase in size. A suggestion of cusp formation may also be seen. At twelve

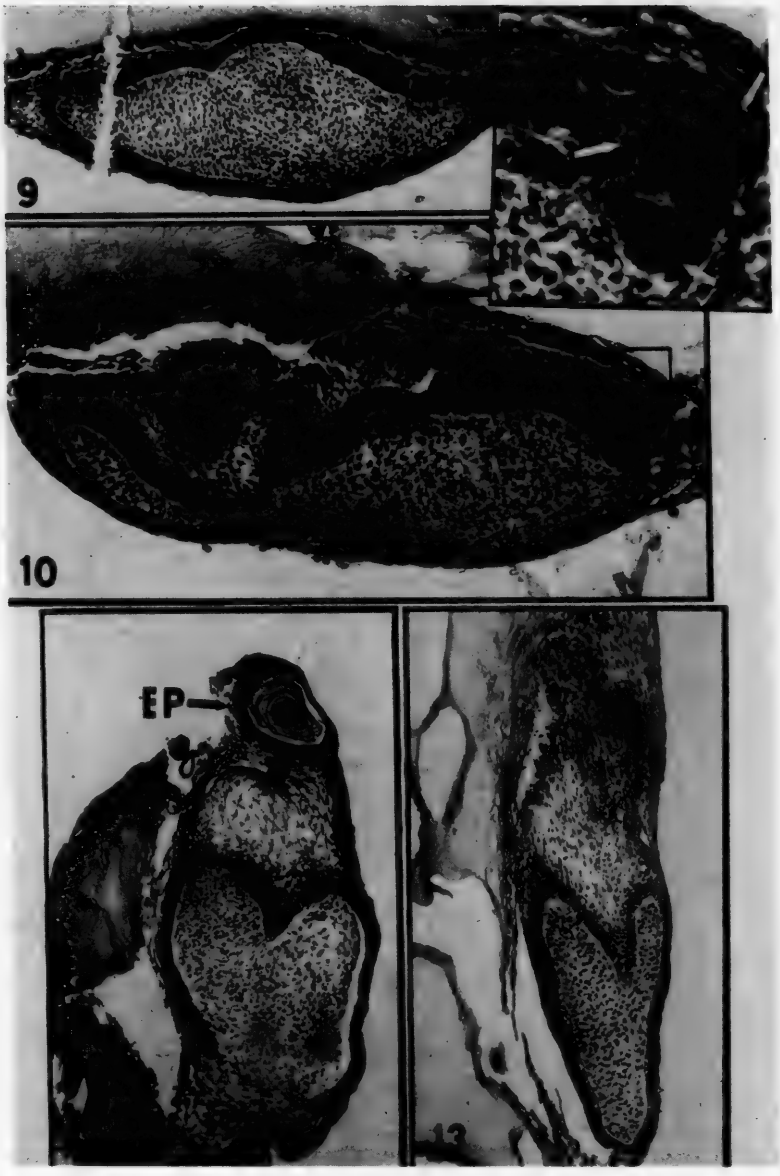


Fig. 9.—Cultivated 19-day tooth germ, follicular wall dissected away. Media same as in Fig. 2. Period of cultivation twelve days. Note mitosis of preameloblasts, survival of cells. Section cut obliquely. Original magnification, ×150, reduced ⅘.

Fig. 10.—Cultivated 19-day tooth germ, follicular wall dissected away. Media same as in Fig. 2. Period of cultivation twenty days. Note fibroblast invasion of central cell area, abnormal cusp development, survival of cells. Section cut obliquely. Original magnification ×315, reduced ⅘.

Fig. 11.—Higher magnification of circumscribed area in Fig. 10. Arrows point to dividing cells. Original magnification ×800, reduced ⅘.

Fig. 12.—Cultivated 18-day tooth germ dissected with follicular wall. Media same as in Fig. 2. Period of cultivation eight days. Note increased invagination of proliferative zone, increase in cusp outline, basement membrane, fibrous invasion of central cell area. *EP*, Epithelial pearl. Section cut buccolingually. Original magnification ×200, reduced ⅘.

Fig. 13.—Cultivated 18-day tooth germ dissected with follicular wall. Media same as in Fig. 2. Period of cultivation 16 days. Note increase in cusp outline, basement membrane, outer tunic showing islands of epithelium with connective tissue invasion of central cell area. Buccolingual section cut obliquely. Original magnification ×200, reduced ⅘.

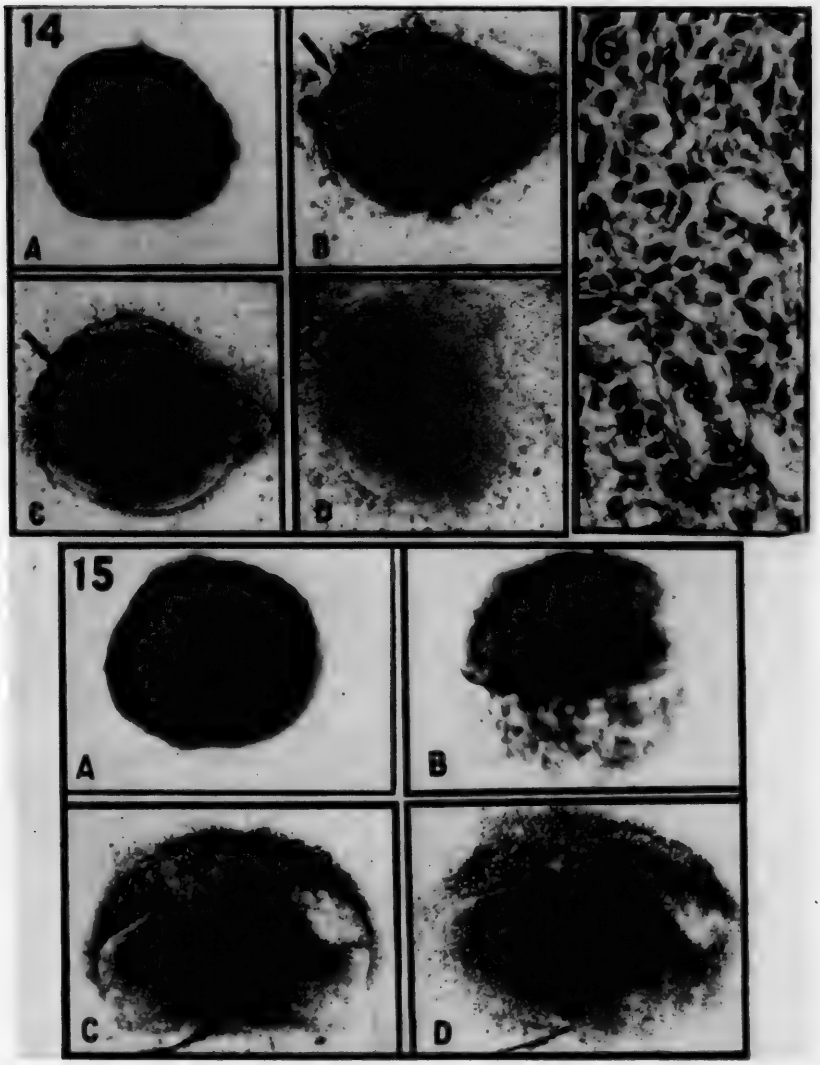


Fig. 14.—Living 19-day tooth germ photographed in Carrel flask showing mesial or distal surface. Same specimen as shown in Fig. 10. *A*, One day after planting; *B*, eight days; *C*, twelve days; *D*, twenty days. Arrows point to cusp outline. Note enamel organ, predentin, growth. Original magnification ×40, reduced ⅓.

Fig. 15.—Living 19-day tooth germ photographed in Carrel flask showing buccal or lingual surface. Same specimen as shown in Fig. 9. *A*, One day after planting; *B*, four days; *C*, eight days; *D*, twelve days. Note enamel organ, predentin, growth.

Fig. 16.—Area of papilla of 20-day tooth germ. Media and period of cultivation same as in Fig. 2. Note degenerating erythrocytes, normal fibroblasts.

days (Fig. 14, *C*), growth is again obvious but much less than observed in the first four days. The tooth germ has increased in size across the narrow diameter. Cusp outline is less obvious. At twenty days (Fig. 14, *D*), there is an increase in size and cusp outline.

Fig. 15 shows a similar development for the tooth germ seen in Fig. 9. One day after planting, it appeared as a round mass similar to that shown in Fig. 14, *A*. Four days later (Fig. 15, *B*), it increased in size, became more translucent, and the formation of three cusps established that the explant was visible from the buccal or lingual surface. At eight days (Fig. 15, *C*), the layer of preameloblasts and basement membrane were readily discernible. Cusp outline, which indicates development, had advanced and there was growth of the explant. At twelve days (Fig. 15, *D*), additional growth occurred. There was deeper invagination of the proliferative zone on the mesial surface. The distal cusp can no longer be seen. From the changes in cusp outline, it is evident that the tooth germ lies obliquely and the distal cusp is deepest in the clot, becoming more obscure as growth continues.

## DISCUSSION

In all experiments, there was an unorganized outgrowth of cells migrating from the explant. It varied in amount. In the main, it consisted of fibroblasts or a mixed outgrowth containing fibroblasts and squamous epithelium.

Observations of explants and sections illuminate the relationship of the outgrowth to the dissection. If the explant includes the follicular wall, the outgrowth will be both fibroblastic and epithelial. The fibroblasts of the follicular wall are undifferentiated and divide readily. Prenatally the follicular wall does not sever the neck of the enamel organ[10] so that these epithelial cells remain in a small area of the surface of the explant. The cells of the neck, which function as part of the oral epithelium, are capable of dividing. On occasion, fragments of oral epithelium may also be retained in the explant. These cells proliferate as unorganized growth, as do the fibroblasts of the follicular wall. The epithelial outgrowth may form epithelial pearls, a process which occurs during rapid proliferation.

Excision of the follicular wall changes the character of the outgrowth. The outer tunic, which differentiates immediately upon formation, loses the ability to divide. These embryonic epithelial cells possess predetermination and proceed toward their developmental goal.[13] They are apparently capable of dividing only to produce proliferative growth, and do not lend themselves to unorganized outgrowth. At the apical area where the fibroblasts form the peripheral cells, there is fibroblastic outgrowth.

Variation in growth and development occurred within the same flask. Each flask contained two tooth germs widely separated in the clot. Both were fixed at the same time. In some cases, even though they originated from the same embryo, variations in development occurred in the same in vitro environment. The amount and type of outgrowth also varied with the flaskmates. As far as could be determined, variations in growth and development were the result of dissection. Exceptions to normal development were rare. In only one case did

abnormal cusp outline occur (Fig. 10). This was difficult to interpret because of the angle of the section.

When the follicular wall was excised, the cultivated tooth germ changed its morphology from eggshaped to a concave disc. It seemed to flatten parallel to the base of the flask, which may explain the translucency after four to six days (Figs. 14 and 15). The development and growth potential of the cells seemed unimpaired despite the gross morphologic alteration. Differences in development with or without the follicular wall were not apparent. Hence from all observations, it seemed desirable to retain the follicular wall.

No specific study was made of the effect of pH on the in vitro culture of tooth germs at this time. It seemed pertinent, however, to report on pH changes as they were observed. At the time of washing and adding supernatant media, the drop in pH was recorded. More reduction was observed in those flasks having greater outgrowth. All flasks were adjusted to pH 7.6 until the next period of washing and adding nutrient fluid.

There is no apparent increase in vascularity in the explant. Capillaries present at the time of planting seemed to survive, but the erythrocytes degenerated (Fig. 16).

The results of in-the-clot cultivation of tooth germs may be studied as development, growth, and survival. Development, manifested by an advance by stages from a lower to a higher state, occurred only in the first period of cultivation. Similar results were reported by Glasstone.[3] The younger explants exhibited more development than the older ones. The preameloblasts exhibited autonomy by continuing to divide, form cusp outline, and differentiate into ameloblasts showing terminal bars and Tomes's processes. The fibroblasts of the papilla formed predentin and differentiated into odontoblasts.

Growth was manifested by cell division in the proliferative zone. In addition, the explants exhibited an increase in size. Glasstone[3, 4] reported shrinkage of the tooth germ, and still mitosis occurred. The pattern of growth did not conform to that which occurs in vivo. With the media used, the rate of growth diminished with the time of cultivation.

Survival occurred in all cultures of dissected tooth germs in which rat embryo extract was used in the clot and supernatant fluid. While the culture of tooth germs in chick embryo extract showed development, degeneration of the deeper cells followed. Glasstone[3] also reported degeneration particularly of the mesodermic cells in which chick embryo extract was used. The survival of cells in rat embryo extract simulates the results of homologous embryonic transplants which survive indefinitely.[14]

Rat molar tooth germs, cultivated in a chicken plasma clot with rat embryo extract of the same age as the explant, survived, grew, and developed. The possibility that growth-stimulating substances arise from the head of the embryo suggests the advisability of its inclusion in future media. Another possibility for further development is the use of older embryo extract at regular intervals. Such investigations are contemplated in an effort to elucidate further information on growth-stimulating media.

SUMMARY

Rat molar tooth germs may be successfully cultivated in Carrel flasks. Rat embryo extract provides a better nutrient media than chick embryo extract.

Using media made from bodies of the same age as the explant, survival, growth, and two days of development occurred.

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# DISSECTION OF RAT MOLAR TOOTH GERMS

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FAULTLESS dissection is a necessary prerequisite to the in vitro culture of tooth germs. They should be excised with the least possible trauma, leaving no torn or shredded tissue at the periphery. Injured tissue may proliferate as unorganized growth rather than undergo fundamental adaptation.[1]

A study of the prenatal stage of odontogenesis of the rat molar[2] provided invaluable aid in the development of the dissection technic. Added to experiences gained by trial and error, a method was developed, which is offered to other investigators in the field.

Losee[3] briefly described a method for dissecting maxillary tooth germs. The best results were obtained at 20 and 21 days' embryonic age. It is not known whether the age of the embryo was recorded from the time of copulation or twenty-four hours after recovery of the plug.[4] If it is the former, Losee's 20-day embryos are equivalent to 19-day embryos in this report.

A total of 304 tooth germs was dissected from rat embryos of 17 to 21 days' embryonic age. The best results were obtained at 19 days. This technic will be described in detail, and modifications for other ages will be mentioned briefly.

The dissection of tooth germs for in vitro culture requires strict adherence to the rules of asepsis. Contamination by the flora of the oral cavity is avoided by using embryos which are sterilely removed in the uterus immediately upon putting the pregnant rat to death. The embryos are then dissected out of the uterus and placed in Tyrode's solution.

Each litter contains from three to fourteen embryos. The heads are severed and kept in a refrigerator at 3° C. until ready for dissection. If permitted to remain at room temperature, the tissues become tacky, and the dissection is more difficult.

The head of the embryo is placed on a sterile glass plate on the stage of a dissecting microscope. A specially constructed glass canopy protects it from air-borne contamination (Fig. 1). The tissue is kept wet with Tyrode's solution during the entire dissection procedure.

The tooth germs lie close to the surface mucosa, and the ridges are in contact. This necessitates opening the mouth before severing the body of the mandible from the ramus. Failure to do so invariably results in injury to either maxillary or mandibular tooth germs. This is done on one side and

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the mouth laid open so that the opposite side may be similarly cut. In this way, it is possible to remove the lower jaw without injuring the tooth germs. The area of the ridges is readily recognized as a bulbous oval eminence (Figs. 2 and 3). The tongue and cheeks are removed from the mandible, which is then cut through the symphysis. These procedures are carried out grossly. The microscope stage and canopy provide protection from contamination.

Fig. 1.—Dissection table. Microscope with glass canopy, lamp, Petri dish containing embryos, hollow ground slides under Petri dish to receive dissected tooth germs, instruments, rack with pipettes, extra glass plates, and hollow ground slides in Petri dishes.

The remainder of the dissection is performed under a magnification of approximately thirty times. The best results are achieved using two Wheeler discission knives so that cutting or holding may be carried out with either hand. The bone is sufficiently soft to be cut with a knife. Cutting from above downward frees the area containing the tooth germs. Meckel's cartilage, which is readily discernible, offers a landmark for excising the inferior portion of the mandible (Fig. 3). At this stage, the buccal and lingual bony plates which arise on either side of Meckel's cartilage are barely joined inferiorly.[2] The two plates of bone are separated and laid open. The oral mucosa acts as a hinge (Fig. 4).

The tooth germs now lie in one-half of the bony encapsulation. They appear as two pearly egg-shaped bodies joined by a constricted portion. These are the first and second molar tooth germs, which are joined by the lamina and enveloped in a fibrous capsule (Fig. 4).

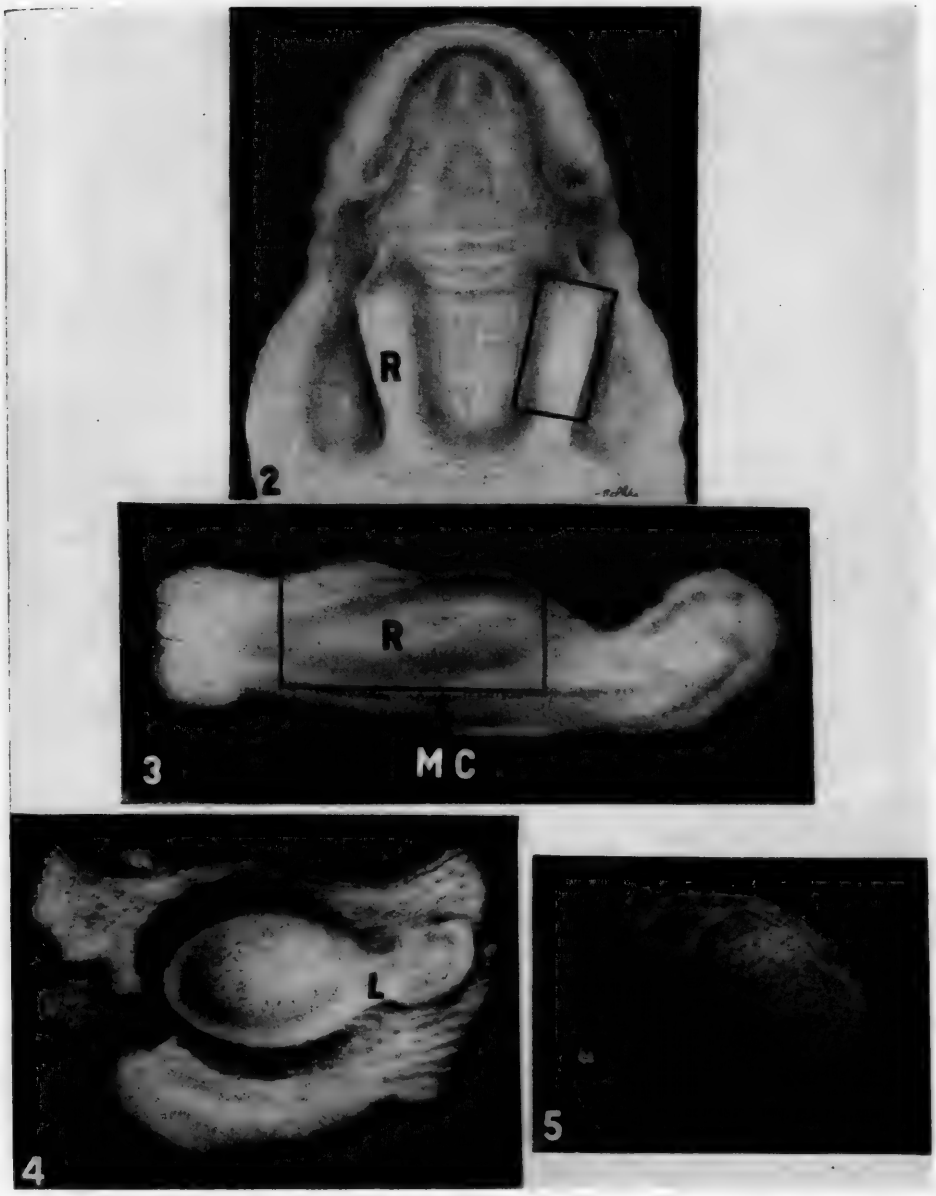


**Fig. 2.**—Palatal view of 19-day rat embryo. *R* denotes ridge containing tooth germs. Lines denote area excised.

**Fig. 3.**—Mandible of 19-day rat embryo. *R* denotes ridge containing tooth germs. Lines denote area excised. *MC* represents Meckel's cartilage.

**Fig. 4.**—First and second molar tooth germs of 19-day rat embryo separated by lamina (*L*) lying in divided bony crypt.

**Fig. 5.**—Dissected tooth germ of 19-day rat embryo.

An incision through the oral mucosa presents the tooth germs in one-half the bony crypt. The next cut is made distal to the first molar tooth germ. The remaining bone is then removed in small sections, which is accomplished by holding the bone with one knife and cutting on the side away from the tooth germ. Small shreds of oral mucosa still attached to the tooth germ are removed in this manner. It is also possible to dissect away the follicle, leaving the enamel organ and papilla. The dissected tooth germs (Fig. 5) are transferred by a pipette to hollow ground slides containing Tyrode's solution until planted

In the maxilla, a similar block of tissue containing the tooth germs is removed (Fig. 2). A thin plate of bone above separates the buccal and lingual plates. This is divided as in the case of the mandibular dissection, and the procedure is the same.

The dissection of 17-day embryos presents more difficulty. The tooth germ is about one-half the size of that found at 19 days. There is little bone formation and encapsulation. The follicle is relatively loose and is more difficult to separate from the surrounding mesenchyme. It is best approached from the apical area, there being no bony crypt to lay back. The dissection is made by removing small pieces of tissue away from the tooth germ. A clean dissection is rarely possible at this stage of development.

At 18 days the dissection is difficult, but good results are possible. The follicle is still not well defined. Bone formation does not lend itself to exposing the tooth germ by dividing the bony crypt. The dissection is best made by excising small pieces of tissue away from the tooth germ. Attempts to separate the bony plates frequently resulted in tearing the follicular wall and injuring the tooth germ.

Between 19 and 20 days' embryonic age, there is a rapid formation and calcification of the bony crypt. At 20 days, the follicular wall is the inner periosteum of the bony crypt. Attempts to divide the bony plates failed. At this stage of development, the best approach is from the occlusal surface, there being no bone in this area. The buccal plate of the bony crypt is first removed. The lingual plate with the tooth germ lying in one-half the bony crypt is reduced to its smallest size. The bony plate is held with one knife, and the second one is used to cut the bone on the side away from the tooth germ. The bone is cut with a rocking motion. By continued removal of small sections of bone the tooth germ is freed.

At 21 days, the removal of the embryos presented an additional problem. The amniotic fluid was viscid and stringy. The tissue also was tacky, and adhered to the knives. Bone growth in the jaws was dense. The technic used at 20 days was followed with considerably less success.

## SUMMARY

The successful dissection of tooth germs is related to the stage of development. The optimum age for dissection is 19 days' embryonic age. At 18 and 20 days, good dissections are possible. At 17 and 21 days, the technic is difficult and results are poor.

Grateful acknowledgment is made to Shirley Glasstone Hughes for preliminary instruction in the technic of dissection of tooth germs.

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# STUDIES ON PHYSICOCHEMICAL PHENOMENA RELATED TO DENTAL CARIES

## I. The Effect of Sugar on the Rate of Diffusion of Hydrogen Ion

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IT HAS already been widely accepted that fermentable sugars play an important role in the development of carious lesions by acting as substrate for oral microorganisms.[1] There is also a possibility of a further role of these substances in addition to that of source of the decalcifying acids. In those regions of the tooth surfaces where food debris is trapped, conditions are frequently such that a saturated solution of the chemical entities of the food material results on or adjacent to the tooth surface. The lower molecular weight members of the carbohydrate class, such as glucose and sucrose, are noted for their extreme water-solubility, and a near-saturated solution of one of these sugars represents an environment quite different from pure water or the usual physiologic media of more moderate concentration. Therefore, it was of interest to examine some of the physicochemical phenomena that may be involved in the carious process in an environment of concentrated sugar solution as compared to water.

It is readily apparent that in so complex a process as the development of the carious lesion at least several physical and chemical processes are in operation under the influence of a large number of simultaneous variables. Rather than attempt to duplicate the natural situation in its entirety, with its concomitant uncertainties, it was decided to evaluate the influence of a particular variable on a specific process in a controlled, isolated system. This paper deals with the relative rates of diffusion of hydrogen ion through a semipermeable membrane from sugar solutions of various concentrations as compared to diffusion from water.

Direction of physical diffusion is determined by the concentration or activity gradients of the individual diffusible particles, except in the few special cases described by Hober.[2] The rate at which this process takes place depends on the nature of the membrane or diffusion barrier and on the nature of the systems on either side of the membrane. In a comparative study using the same membrane and varying the components on one side of the membrane or the other, these variations become the controlling factor. It is obvious that the addition of extraneous material, such as sucrose in high concentrations, will influence the be-

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havior of the diffusible ions, but the effect of this influence has not been demonstrated. If, in this case, one considers a membrane permeable to hydrogen ion and impermeable to the sucrose molecule, three possibilities arise.

1. The rate of diffusion of the diffusible ions may be increased since, in a concentrated sugar solution, the actual volume of free solvent is less than it would be in an equal volume of water due to the space occupied by the sucrose molecules and to the strong tendency of polyhydric molecules to bind water molecules through hydrogen bridges. This would mean that the actual concentration of hydrogen ion is greater in the system with sugar than would be expected from the normality number.

2. The rate of diffusion of the diffusible ions may be decreased due to the operation of one or more of the following factors: increased viscosity of the solution, competition for the diffusion sites in the membrane by an increased number of molecular species, and osmotically induced flow of solvent counter to the direction of diffusion of the diffusing ions.

3. The accelerating variables may balance the impeding variables so that no change would be observed.

## EXPERIMENTAL

In order that extraneous variables and experimental error be kept to a minimum, and to facilitate a statistical analysis of the experimental data, it was necessary to establish and adhere rigidly to a definite schedule of procedure. The aim of this work was to arrive at relative values within the experiment rather than at absolute physical values. To this end, each series was divided into four treatments, diffusion of lactic acid from water, from 0.5 M sucrose, from 1.0 M sucrose, and from 1.5 M sucrose. These four conditions were studied twice on each of six diffusion membranes so that effects due to aging of the individual bag or to differences in different bags would be cancelled out. In order to evaluate the effect of the lactate ion, the whole set of experiments was repeated with 0.001 N KCl replacing distilled water as the counter-solvent.

Diffusion was carried out from sacks made of collodion, Cenco, U.S.P. It was found that membranes prepared from collodion flexible were highly impermeable even to hydrogen ions. All sacks were made in the same glass mold, a round-bottom tube 35 mm. by 205 mm., by pouring a sufficient amount of collodion into the tube to cover the bottom to a depth of 20 mm. and then pouring the collodion out while rotating the tube in such a manner so as to leave an even coating on the walls. The tube was rotated in the hands at an angle of forty-five degrees from horizontal, mouth down, until the ether had evaporated. It was then clamped vertically, mouth down, for twenty minutes, washed well with distilled water, removed from the mold, and fastened to a glass neck of suitable size with several turns of fine cord. A bag so prepared was found to be quite permeable to hydrogen ion and only slightly permeable to sucrose, so that by the end of the experiment only an insignificant amount of sucrose had left the bag.

Stock solution of N lactic acid was prepared from 85 per cent lactic acid and was checked by titration. Stock solution of 2 M sucrose was prepared from sucrose, reagent grade, and stored at 5° C.

The experiments were carried out in an apparatus designed and built for this work. This all-glass apparatus, shown in Fig. 1, allowed for efficient stirring inside and outside of the bag without the danger of damage to the membrane by the impeller blades. Further, this allowed for a small volume ratio between the two solutions (3:1) while affording room for the necessary equipment.

Changes in hydrogen ion concentration in the outer solution were followed with a line-operated continuous-reading Coleman pH meter, Model 18. The whole apparatus was maintained at 25° ± 0.1° C. in a Sargent constant temperature bath.

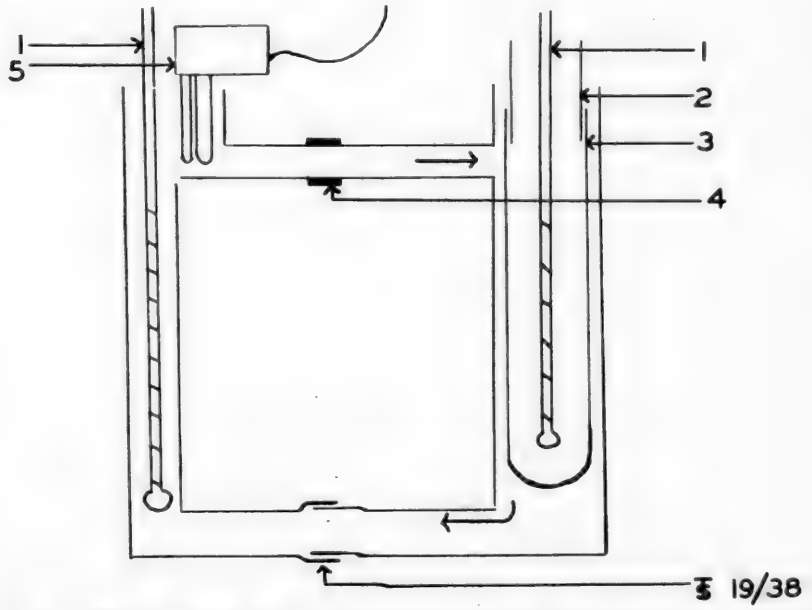


Fig. 1.—Diffusion apparatus. *1,1*, Power-driven, helical thread stirrers; *2*, glass-neck to hold bag; *3*, collodion diffusion bag; *4*, glass-to-glass junction closed by rubber tubing; *5*, electrode head and electrodes.

A freshly prepared collodion sack was filled with 130 ml. of 0.001 N lactic acid, prepared by suitable dilution of an aliquot of stock N lactic acid and previously brought to temperature. The outer surface of the bag was wiped dry and checked for leakage. It was then clamped in place in the apparatus, the stirrers and electrodes were properly mounted, and 400 ml. of distilled water, previously adjusted to pH 6.50 with a few drops of dilute NaOH, was rapidly added to the apparatus. Readings of pH of the outside solution were taken every minute for twenty minutes, followed by two additional readings at five-minute intervals. At this time the apparatus was dismantled, rinsed well with distilled water, and the process repeated with 0.001 N lactic acid in 0.5 M

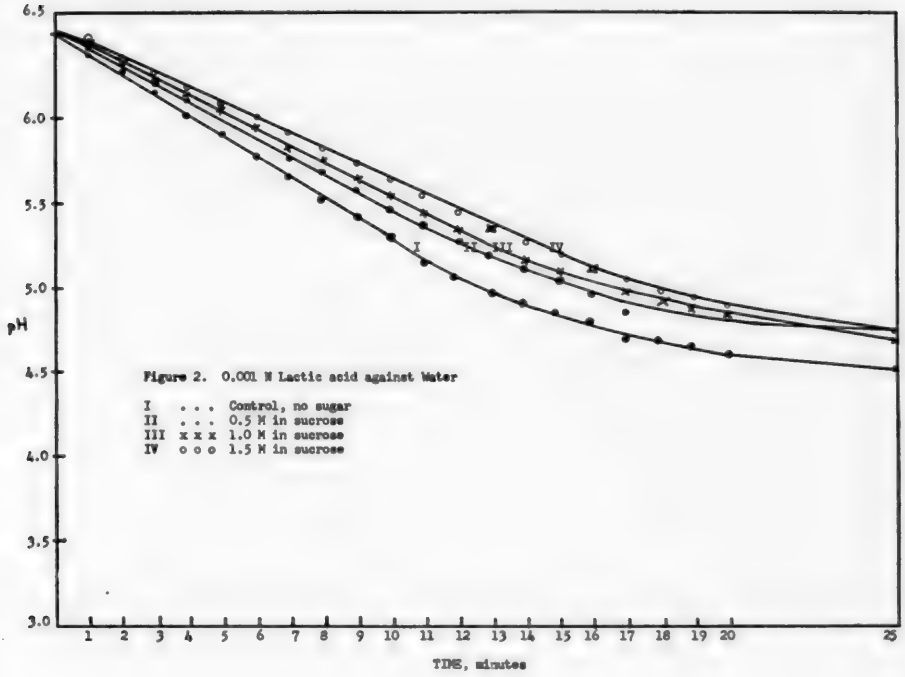


Figure 2. 0.001 N Lactic acid against Water

I . . . Control, no sugar
II . . . 0.5 M in sucrose
III x x x 1.0 M in sucrose
IV o o o 1.5 M in sucrose

Fig. 2.

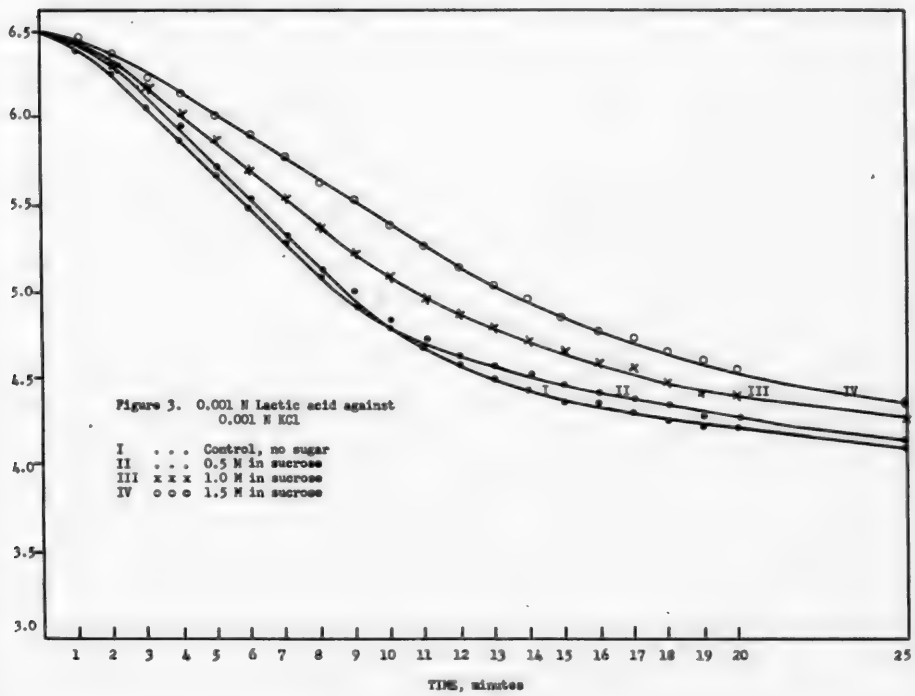


Figure 3. 0.001 N Lactic acid against 0.001 N KCl

I . . . Control, no sugar
II . . . 0.5 M in sucrose
III x x x 1.0 M in sucrose
IV o o o 1.5 M in sucrose

Fig. 3.

sucrose. This whole procedure was again repeated with 0.001 N lactic acid in 1.0 M sucrose and with 0.001 N lactic acid in 1.5 M sucrose. After this fourth run, the collodion sack was soaked in distilled water for thirty minutes in addition to the usual washing, and the four experiments were repeated.

In a similar series of experiments, 0.001 N KCl adjusted to pH 6.50 was used in place of distilled water as the outer solution.

## RESULTS AND DISCUSSION

The results of these experiments are shown in Figs. 2 and 3 as plots of pH of outside solution versus time. Each point on the curves represents the mean of twelve separate determinations. From these graphs, it is clearly seen that the rate of diffusion of acid is inversely proportional to the concentration of the sugar solution from which it is escaping.

Consideration of the relative sizes and mobilities of the hydrogen ion and the lactate ion leads to the possibility that in the diffusion of lactic acid, the diffusion coefficient of the lactate ion may be the limiting factor. When the diffusion process is carried out against pure water, it is necessary for equivalent amounts of cation and anion to pass the barrier at any instant in time to maintain electrical neutrality. However, if there are diffusible electrolytes on both sides of the membrane, electrical neutrality can be maintained by either the simultaneous diffusion of both cation and anion from the first side to the second, or by exchange of cations and/or of anions between the two sides. The latter set of conditions is represented in the second series of experiments in which the distilled water was replaced by KCl at the same concentration as the lactic acid inside the bag. Under these conditions it would be possible for $K^+$ to enter the bag to replace the $H^+$ which leaves, and thus the diffusion rate of the hydrogen ion would be freed of the limiting effect of the lactate ion. This is more analogous to in vivo conditions, where distilled water cannot be expected to exist as the counter-solution to a diffusion process. Comparison of Curve I of Fig. 2 with Curve I of Fig. 3 shows that hydrogen ion does diffuse faster against the salt solution than against distilled water, indicating the limiting effect of the lactate ion.

TABLE I

SUMMARY OF ANALYSIS OF VARIANCE

| ITEM | DEGREES OF FREEDOM | MEAN SQUARE | "F" | P* |
|---|---|---|---|---|
| Main plot | 3 | 109.875 | 28.311 | $<0.01$ |
| Sucrose concentration | | | | |
| Time | 20 | 491.637 | 126.677 | $<0.01$ |
| Interaction | 60 | 1.413 | 0.364 | $>0.05$ |
| Conditions | 1 | 549.589 | 141.610 | $<0.01$ |
| Error | 83 | 3.881 | | |
| Total | 167 | | | |

*P is the probability for the observed difference to have occurred by chance.

In order to fully evaluate the statistical significance of the experimentally observed differences, the data for the first twenty minutes of each experiment were submitted to an analysis of variance according to the methods presented

by Snedecor.[3] A summary of the pertinent statistical values is given in Table I. Since the experiments were designed primarily to ascertain the effect of sucrose on the diffusion rates, all other variables, with the exception of the condition of presence or absence of KCl, are collected under one item labelled Error. The effect of KCl is shown under Conditions. From the table it can be seen that the probability of the observed differences being due to chance is less than 1 part in 100.

Although the acid used here was lactic acid, it is obvious that hydrogen ion is the same irrespective of its source. Likewise, in the carious process the decalcifying agent is apparently hydrogen ion although the nature of the acid anions may be as yet not fully determined. Criticism of the chemicoparasitic theory of caries has been based in part on the failure of acid per se, placed on the surface of a tooth in vitro, to reproduce the lesion found in clinical caries. In this work, we have shown that in an environment similar to that which may exist on the surface of a tooth in vivo, that is, acid in the presence of a high concentration of sugar molecules, the physical diffusion of hydrogen ion is modified. Thus, it is quite possible that other seemingly simple physicochemical phenomena which may be involved in the carious process may also be modified from the behavior noted in dilute systems.

## SUMMARY

1. The rate of diffusion of hydrogen ion through a semipermeable membrane was studied under various conditions of sugar concentration.

2. It was found that the rate of diffusion of acid was inversely proportional to the concentration of sugar in the acid solution.

We wish to acknowledge the kind assistance of Dr. S. C. Harris, Northwestern University Dental School, in the statistical treatment of the experimental data.

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# STUDIES ON PHYSICOCHEMICAL PHENOMENA RELATED TO DENTAL CARIES

## II. The Effect of Sugar on the Rates of Diffusion of Calcium and Phosphate Ions

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THERE is considerable evidence to indicate that sugar is important to the formation of carious lesions. One major role of sugar, that of providing a substrate for the formation of acids, has been adequately explained; there is, furthermore, the probability that sugar may also be of considerable importance because of its physical properties. The living tooth is known to be a semipermeable membrane, and ions and small molecules may pass through this barrier. Because sugar has a high molecular weight and because it is extremely soluble, sugar can form solutions of high molecular concentration, and for this reason may cause a modification of the diffusion of particles through the enamel of the tooth. Previous work has shown[1] that concentrated sugar solutions do modify the passage of hydrogen ions through collodion membranes; it was found that sugar causes a marked decrease in the rate of penetration. If this information concerning the effect of sugar on the diffusion of hydrogen ions through collodion membranes also applies to the passage of hydrogen ions through the enamel layer, it would seem that the presence of sugar on the tooth surface should decrease the progress of the carious lesion.

Direct application of these results cannot be justified, however, because of other variables. The natures of the membranes are obviously not the same. In the tooth, the hydrogen ion reacts with the membrane, and hence it will penetrate only until the reaction takes place. Furthermore, once a hydrogen ion has displaced a calcium ion of the tooth, the soluble products of reaction may accumulate and interfere with diffusion.

In order to gain information on the latter phase by a simple system, an experiment was set up so that a suspension of tricalcium phosphate was separated from an acid solution by a semipermeable collodion membrane. A measurement was made of the rate at which the solubilized calcium and phosphate ions diffused out of the membrane under various conditions involving the presence or absence of sugar. Under conditions of the experiment, the acid must diffuse through the membrane and attack the insoluble phosphate; then the products of reaction must diffuse back through the membrane. Under one set of conditions, the direction of hydrogen ions was the same as the osmotic flow, while the products of reaction were against it. Under another set of conditions, the migration of the calcium and phosphate ions was in the same direction as the

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osmotic flow, while the direction of the hydrogen ions was against this flow. The migration of the ions could be altered by the presence of sugar because of the viscosity of the solutions, competition of additional molecules for diffusion sites, the osmotic flow of water, the tendency of the sugar to complex with calcium ions, and the tendency of sugar to bind water. The over-all rate of diffusion could be either decreased or accelerated depending on the relative importance of the previously mentioned variables.

## EXPERIMENTAL

These experiments were carried out in the diffusion apparatus described in the first paper of this series,[1] and the diffusion sacks were prepared from collodion U.S.P., Cenco, as before. The temperature was maintained at 25° C. ± 0.1° C. in a constant temperature bath.

These studies consisted of a series of three treatments. In Treatment I, tricalcium phosphate, chemically pure, was suspended in water inside the collodion bag, and the rest of the apparatus was filled with 0.1 N hydrochloric acid. For Treatment II, the tricalcium phosphate in the bag was suspended in M sucrose, and in Treatment III, the tricalcium phosphate in the bag was suspended in water and the acid was dissolved in M sucrose. Unless all three treatments were carried out on the same bag, all data for that bag were excluded from the final comparison. This series of three treatments was carried out on each of three collodion diffusion bags.

*Procedure.*—A mixture of 3 Gm. of tricalcium phosphate and 130 ml. of water was added to a freshly prepared collodion diffusion sack. The bag was checked for leakage and then clamped in place in the larger limb of the diffusion apparatus. The stirrers were positioned properly and, with the stirrers in operation, 400 ml. of 0.1 N hydrochloric acid was added rapidly to the diffusion apparatus. At five-minute intervals for one hour, two 1 c.c. samples were removed from the outer solution in the system for analysis for calcium and phosphorus. At the end of one hour, the apparatus was dismantled, cleaned thoroughly, and the experiment repeated in an identical manner, except that the tricalcium phosphate was suspended in 130 c.c. of M sucrose solution. At the end of this experiment, the entire process was repeated again with the tricalcium phosphate suspended in water and the 0.1 N hydrochloric acid solution in M sucrose.

Calcium was determined by direct titration with a sequestering agent according to the method of Biederman and Schwarzenbach.[2] Phosphorus was determined by a modification of the Fiske and Subbarow method.[3]

In one case, Treatment I was repeated on the diffusion bag after the other treatments had been carried out in order to determine the effect of the order of treatment on the results. The order of treatment had no effect on the results.

## RESULTS

The data from the series of experiments in which the different treatments were compared are collected in Tables I and II and are tabulated as the mean values of the triplicate series.

TABLE I

MEAN CALCIUM (MILLIMOLS PER MILLILITER)

| TIME (MIN.) | TREATMENT | | |
|---|---|---|---|
| | I | II | III |
| 5 | 0.0017 | 0.0017 | 0.0011 |
| 10 | 0.0040 | 0.0034 | 0.0033 |
| 15 | 0.0060 | 0.0060 | 0.0049 |
| 20 | 0.0087 | 0.0075 | 0.0065 |
| 25 | 0.0107 | 0.0094 | 0.0078 |
| 30 | 0.0132 | 0.0112 | 0.0096 |
| 35 | 0.0160 | 0.0134 | 0.0119 |
| 40 | 0.0182 | 0.0155 | 0.0140 |
| 45 | 0.0205 | 0.0168 | 0.0158 |
| 50 | 0.0234 | 0.0192 | 0.0181 |
| 55 | 0.0254 | 0.0207 | 0.0196 |
| 60 | 0.0271 | 0.0221 | 0.0223 |

TABLE II

MEAN PHOSPHORUS (MILLIMOLS PER MILLILITER)

| TIME (MIN.) | TREATMENT | | |
|---|---|---|---|
| | I | II | III |
| 5 | 0.000248 | 0.000357 | 0.000194 |
| 10 | 0.000757 | 0.000893 | 0.000588 |
| 15 | 0.00113 | 0.00194 | 0.00102 |
| 20 | 0.00214 | 0.00210 | 0.00188 |
| 25 | 0.00285 | 0.00288 | 0.00226 |
| 30 | 0.00374 | 0.00330 | 0.00299 |
| 35 | 0.00490 | 0.00398 | 0.00391 |
| 40 | 0.00594 | 0.00476 | 0.00476 |
| 45 | 0.00700 | 0.00541 | 0.00571 |
| 50 | 0.00839 | 0.00642 | 0.00632 |
| 55 | 0.00930 | 0.00700 | 0.00736 |
| 60 | 0.01029 | 0.00761 | 0.00827 |

The data presented in Tables I and II are summarized in the curves shown in Figs. 1 and 2 in which the mean values of the concentrations of the element under consideration are plotted against time.

The mean values for the Ca/P ratios in the various treatments are presented in Table III. In considering this table, it must be remembered that these values have a very low order of precision. The calculated value of this ratio for tricalcium phosphate is 1.5.

TABLE III

CALCIUM/PHOSPHORUS RATIO (MOLE/MOLE)

| TIME (MIN.) | TREATMENT | | |
|---|---|---|---|
| | I | II | III |
| 5 | 6.85 | 4.76 | 5.66 |
| 10 | 5.28 | 3.81 | 5.61 |
| 15 | 5.30 | 3.09 | 4.80 |
| 20 | 4.15 | 3.50 | 3.45 |
| 25 | 3.75 | 3.26 | 3.45 |
| 30 | 3.53 | 3.39 | 3.21 |
| 35 | 3.27 | 3.36 | 3.04 |
| 40 | 3.06 | 3.26 | 2.94 |
| 45 | 2.93 | 3.10 | 2.76 |
| 50 | 2.79 | 2.99 | 2.86 |
| 55 | 2.73 | 2.96 | 2.66 |
| 60 | 2.63 | 2.90 | 2.69 |

## DISCUSSION

It can be seen from Figs. 1 and 2 that under the conditions of these experiments the rates of diffusion of calcium and of phosphate ions are decreased by the presence of sugar. This effect is observed in the case in which sugar is part of the environment of the insoluble phosphate salt and in the case in which the sugar is a part of the environment of the acid. An analysis of variance for

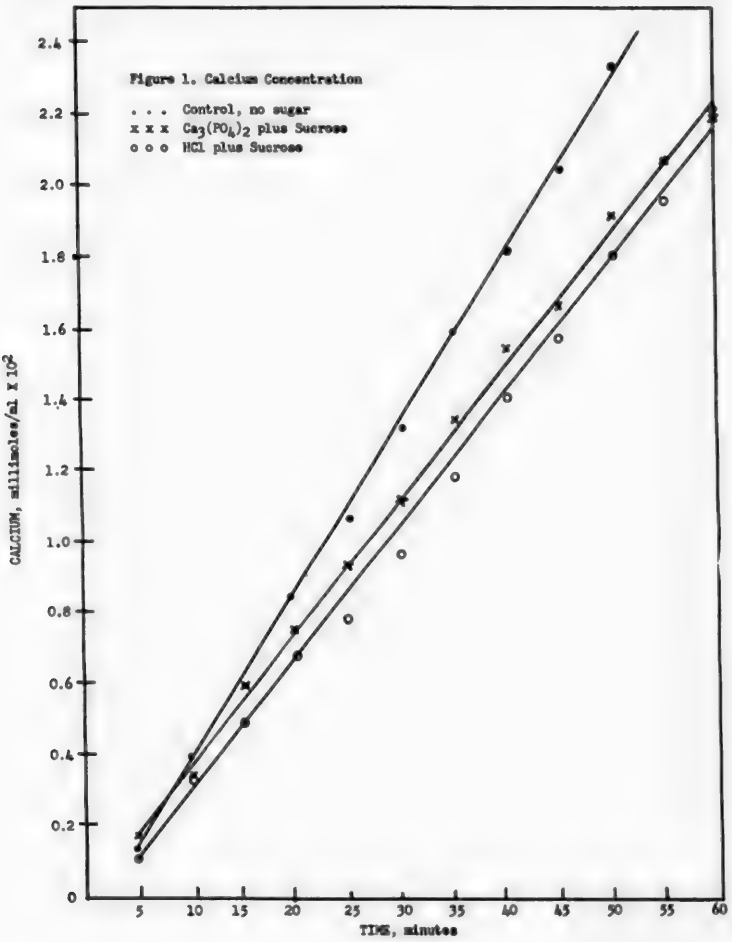


Fig. 1.

the data for calcium shows that the results are significant beyond the 0.01 level. A similar analysis for the data for phosphorus for time = 30 and greater also shows significance beyond the 0.01 level.

The Ca/P ratios showed no significant difference due to the various treatments when the data were treated by the same methods used previously. In each case, the change in this value with time was the same within the error

of the determinations. Therefore, the effect of the sugar is being exerted in a similar manner on both ions, which would disprove a chemical mechanism for this behavior. Further, the fact that the Ca/P ratio is different from the expected calculated value of 1.5 and is not changed by the sugar indicates that the unequal diffusion of cation and anion observed here is a function of the ions and their undissociated salt and not of the medium in which the diffusion occurs. The negative wall charge of the collodion membrane is probably an important factor in causing the slower diffusion of the anion.

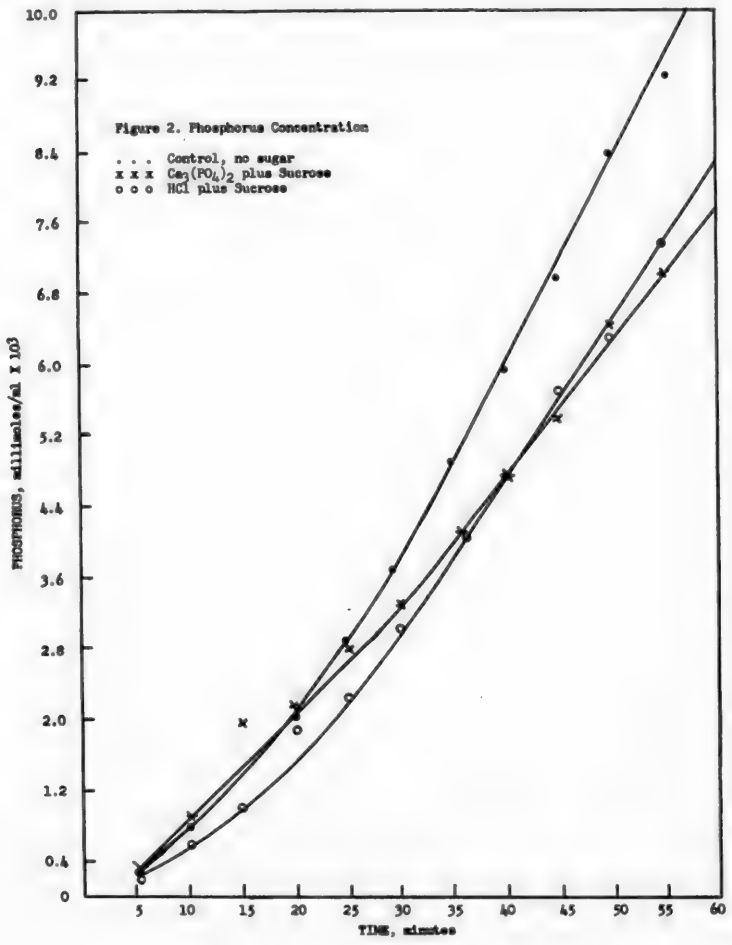


Fig. 2.

The results of these experiments, as well as those of the previous studies on hydrogen ion alone, show that a physical process, diffusion, exhibits a decreased rate in the presence of moderately concentrated sugar solutions. Whether or not

these data may be directly applied to an explanation of the carious process is questionable. More data on more complex systems are needed before the effect of sugar solutions on permeability of tooth enamel can be explained.

## SUMMARY

A study was made of the effect of sugar on the rate of diffusion of calcium and phosphate ions through a semipermeable membrane. The calcium and phosphate ions were generated by exposing tricalcium phosphate suspension to attack by hydrogen ions. It was found that the rates of diffusion of calcium and phosphate ions were decreased in the presence of M sucrose solution. It was found that the initial rate of appearance of calcium was higher than that of phosphate, but that the Ca/P ratio approached the theoretical value of 1.5 with time.

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# THE FORMATION OF EARLY CARIOUS LESIONS IN DENTAL ENAMEL

## PART I

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PRIOR to the development of simpler methods of preparing decalcified sections of enamel, the knowledge of the shape of carious lesions within the enamel was based principally on an examination of longitudinal ground sections of teeth by transmitted light.

For several reasons it seemed likely that useful information could be obtained by examining lesions by incident light microscopy. First, it was felt that in preparing a highly calcified substance for examination it is unwise to subject it to chemical reagents which themselves may be capable of producing changes similar to those believed to occur in the carious lesions. In other words, because attack by acid plays a part in the formation of a carious lesion, to subject either carious or sound enamel to decalcification by acid might lead to confusion between the results of the naturally and of the artificially applied acid. Second, the use of thin ground sections for examination by transmitted light allows an examination of a lesion to be made in only one plane. Third, it is extremely difficult to avoid production of artefacts in the preparation of such sections. The use of material prepared for examination by incident light obviates a number of these difficulties. The lesion need not be subjected to any chemical reagents, and structural relationships are preserved by means of the firm, binding material surrounding the specimen; also, the bulk of the tooth need not be sacrificed. By careful polishing, the production of artefacts can be reduced to a minimum and, when desired, it is possible to examine the lesion in a number of different planes, including different levels of the enamel in a tangential plane.

### THE EXAMINATION OF LESIONS BY INCIDENT LIGHT

*Materials and Methods.—*

1. *Selection of carious material:* Freshly extracted teeth were washed in water and dipped momentarily into hydrogen peroxide in order to remove any loose debris. The teeth were allowed to dry in air and were then examined by means of a dissecting microscope. The basis of selection of the lesions was wholly clinical. Teeth with early carious lesions on an approximal surface were selected. (The phrase "early carious lesion" is used to denote a lesion in which there was no break in surface continuity and, on splitting the tooth, no great involvement of the dentin. The lesions were recognized by a change

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from the normal translucency of the enamel to opaque, white, or brown areas, by their loss of polish, and by their position and shape.)

2. *Preparation of material:* Following the technic of Gustafson[1] and of Jaccard and Grosjean,[2] the coronal portion of the tooth was removed by grinding until the lesion lay in close proximity to the ground surface. By this means it was possible to establish with some accuracy the plane in which the lesion would ultimately be polished and examined. The roots of the tooth were then removed, leaving the lesion surrounded by some sound enamel and supported by dentin. The specimen was fixed to a glass slide with a very small amount of Canada balsam, in order to hold it in the correct position. Next, a small metal cylinder approximately three-fourths inch in diameter was placed around the specimen and packed with plastic wood, thus embedding it. When the plastic wood has set, it had usually shrunk sufficiently to enable the metal cylinder to be easily removed and, after separation from the glass slide, the preparation was ready for polishing. The embedded specimen was carefully examined, lest any change in position had occurred during the embedding. The relatively large bulk of the plastic wood ensured that the surface of the specimen could be polished in the plane selected. The surface was reduced by the use of four grades of fine emery paper (Durex Sil No. 0, 00, 000, and 0000), and polishing on each type of paper was controlled by examining the specimen by means of a dissecting microscope. All papers were used in a dry state. Only light pressure was applied at any stage, and the surface was moved slowly across the papers. Originally, after treatment with the Grade 0000 paper, the specimens were polished by stroking them gently on a moist polishing pad impregnated with heavy oxide of magnesium. Because this process tended to embed the powder in the lesion, it was discarded. All polishing was done by hand in order that the generation of heat could be controlled as much as possible, and the emery papers were always kept on a hard plane base so that no artificial reliefs would be introduced. Plastic wood appeared to be the most satisfactory embedding agent: it set without generation of heat, and its main chemical solvent, acetone, appeared to have no effect on the enamel.

3. *Staining of material:* Dyes were sought which would give a specific reaction with some particular constituent of the enamel and would undergo a color change during the staining process, so that any resultant pigmentation would be due to chemical reaction with that constituent of the enamel and not merely an artefact produced by absorption of the dye. Also, stains were sought which could be used in an aqueous solution in order to minimize any harmful effect on the enamel.

Alizarin red S as a vital stain and as an aid in the examination of bony structures has been used for some time.[3-7] It gives a yellow solution in water, and when in contact with metallic hydroxides it forms insoluble pigments which are termed lakes. Schour, Hoffman, Sarnat, and Engel[5] quoted Gottlieb as stating that he believed it to have a specific chemical affinity for calcium, Cameron as stating that the coloring in hard tissues is due to the

ability of the alizarin to form a dye lake with calcium and that it is nearly a specific dye for calcium, and Proell as stating that alizarin stains free calcium. Lison[8] agreed that calcium salts are stained, but believed that some other salts, such as aluminum and iron, can also effect the alizarin laking. In relation to tooth substance and bone, however, it would appear that alizarin red S can be accepted as a specific stain for free calcium ions. This was checked roughly by submitting some partially and some completely decalcified sections of bone to the action of the dye. The completely decalcified sections showed only slight yellowish coloration, while the partially decalcified sections contained areas of bright red staining. Tests were also carried out with salts of barium, magnesium, calcium, and sodium to determine the specificity of the alizarin. It was found that, although there was a distinct color change if the dye solutions were made alkaline, and also some color change in solutions containing ions other than calcium, the color which formed when calcium ions were present was quite distinctive, and warranted the assumption that the presence of the bright red color indicated the presence of free calcium ions.

It was also decided to stain lesions with some neutral dye in order that the general outline of the lesions might be observed. To this end, numerous specimens were stained with methyl green, which had been used by Frisbie, Nuckolls, and Saunders[9] to test the rate of penetration of fixatives into enamel.

When using alizarin red S, it was found to be equally satisfactory to submit the specimens to a filtered, saturated 2 per cent aqueous solution of alizarin red S either before or after the specimens had been prepared for examination. The period of immersion in the dye varied, but usually was twenty-four hours. When a specimen had been polished prior to staining, it was again very lightly polished after staining in order to remove any surface deposit.

In preparing specimens for staining by methyl green, the surrounding normal enamel and dentin were first reduced to a minimum, and the specimens were then placed in a saturated aqueous solution of methyl green. Initially some specimens were prepared by placing them in a 10 per cent formol saline solution to which had been added sufficient methyl green to saturate the solution. Specimens were left in this medium for up to eight weeks. After washing, all specimens were mounted and polished. No differences were observed in sections prepared by either of the methods.

4. *Examination of the enamel lesions:* The site and nature of the lesions were noted prior to the preparation for examination and again after polishing. Microscopic examination was then carried out by incident light using either a Zeiss Epi-condenser or a Leitz Ultropak.

The Zeiss Epi-condenser was used primarily for investigation of lesions under low magnification, and the Leitz Ultropak was used where magnifications up to ×900 were required, as alternative apparatus was not available.

*Results.*—Before presenting the results, it should again be emphasized that all specimens that were examined were of early carious lesions which, at the

most, extended only a very short distance beyond the dentinoenamel junction, and that they represented only one particular type of lesion in that they occurred on the approximal surfaces of the teeth (thus corresponding to the frontal caries of Gottlieb).

1. *Lesions stained with methyl green:* It was found that even after immersion in formol saline solution and methyl green for periods up to eight weeks, penetration of the dye into the enamel occurred only in the carious area. Any underlying dentin was fully stained, but the staining ceased at the dentinoenamel junction. The lesions developed colors ranging from green to deepest violet, but the blue shades were predominant. Examination of lesions with the unaided eye and at low magnifications merely confirmed the fact that the carious enamel and dentin were the only substances which allowed the dye to penetrate them. There were surface deposits of dye on the normal enamel, but these were readily removed by gentle washing, and the lesions stood out as bright-bluish areas surrounded by normal white enamel. The reactions of the various components of the enamel to the spread of the carious process seemed to vary but little, and the following descriptions give a composite picture which could be applied to any of the lesions examined.

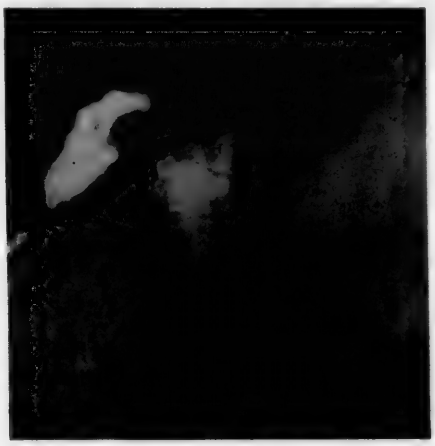

Fig. 1.—Longitudinal section of early carious lesion stained with methyl green. The classical conoidal shape of the lesion can be seen, and it can be noted that the lesion ends in prolongations penetrating along Hunter-Schreger lines. (Magnification, ×37. Incident light.)

(a) The shape of the lesion depended on the plane of section. In *longitudinal section* (that is, a section through the long axis of the tooth), the outline of the lesion conformed to the generally accepted description of a cone, having its base at the enamel surface and its apex at or near the dentinoenamel junction (Fig. 1). Often the apex of the cone ended in finger-like processes which penetrated farther into the enamel and appeared to bear a definite relationship to the Hunter-Schreger lines. In all cases, the margins of the lesion were well defined. They seemed to be influenced greatly by the direction of the enamel prisms and the direction of the striae of Retzius. The

margins of the lesion and the direction of the prisms were, for the most part, parallel, particularly at the base of the lesion. At times, however, the margin of a lesion would be seen to be interrupted where it abutted upon a stria of Retzius so that the margin would then run parallel to the stria.

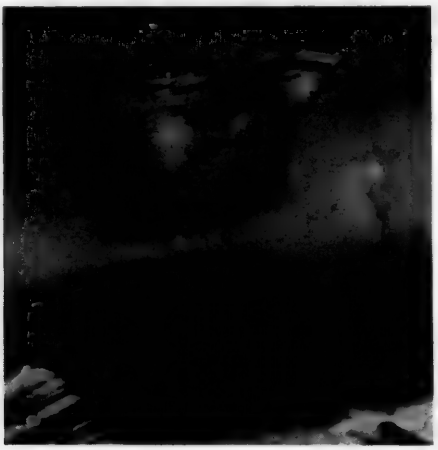

Fig. 2.—Transverse section of early carious lesion stained with methyl green. The variation in shape from traditional conoid form can be seen, and relation of outline of lesion to the striae of Retzius is well marked. The amorphous nature of the majority of enamel in center of lesion can be noted. (Magnification, ×37. Incident light.)

Fig. 3.—Higher magnification of the lesion shown in Fig. 2. Portion of enamel in lesion has broken away, but it has been held approximately in position by binding material of preparation. It can be noted that line of cleavage is along the striae of Retzius. (Magnification, ×75. Incident light.)

In *transverse section* (that is, a section at right angles to the long axis of the tooth), the lesion had a very different appearance (Figs. 2 and 3). Here the margins of the lesion were almost parallel, but again conforming to the direction of the enamel rods. In areas where the margins were not parallel,

there was a "steps and stairs" effect. The innermost limits of the lesion extended over a broad area without any signs of an arrowhead formation, and bore a relationship to the striae of Retzius. In a very shallow lesion, this relationship was even more obvious, and the lesion could be seen to be bounded by walls which were parallel to the long axis of the enamel prisms,

Fig. 4.

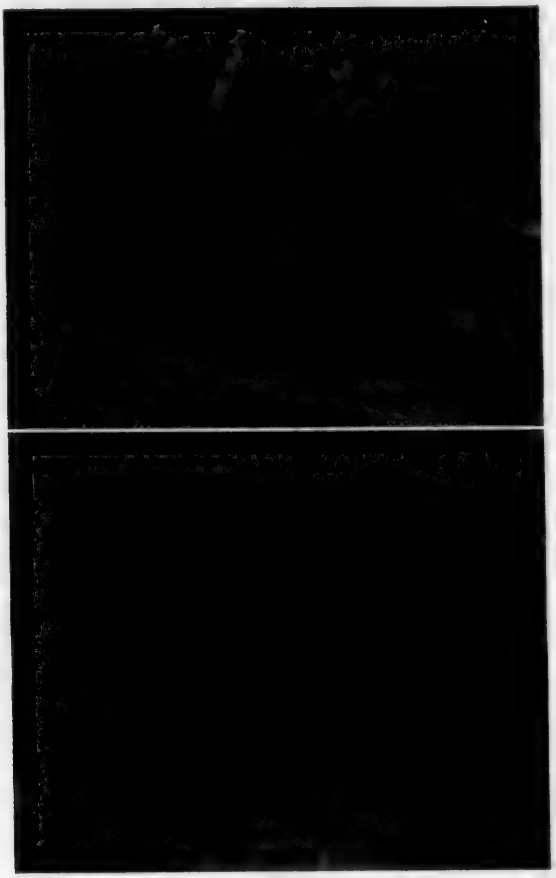

Fig. 5.

Fig. 4.—Higher magnification of base of lesion shown in Fig. 2. Relation of lesion to the striae of Retzius can still be seen and, in lower parts, initial staining appears to be limited to areas surrounding the prisms. (Magnification, ×440. Incident light.)

Fig. 5.—Higher magnification of side of lesion shown in Fig. 2. Here, complete involvement of enamel prisms can be seen. (Magnification, ×440. Incident light.)

the inner limit of the lesion being parallel to the enamel surface (and therefore parallel to the striae of Retzius). In other words, the general shape of the entire lesion was that of a wedge. In older lesions the loss of structural integrity seemed greatest at the center.

(b) Concerning the appearance of the components of the enamel (Figs. 4 and 5), in the stained lesions one of the most obvious features in both

transverse and longitudinal sections was the fact that the direction of the margins of the lesions almost invariably bore a constant relationship to the direction of the enamel prisms. The use of higher magnifications, however, together with the examination of tangential sections (that is, sections parallel to the affected surface of the tooth) disclosed that whereas the central and more superficial portions of the lesions usually were rather amorphous, the stain had not been accepted by the substance of the prisms in the deeper parts of the lesion. The only visible change in the prisms themselves was an appearance of segmentation, that is, the prisms were regularly banded by thin, bluish green lines, the remainder of the prisms remaining a dull white. The reason that the direction of the prisms was still obvious in these areas was that the space between the prisms (that is, either the interprismatic substance or the prism sheaths, or both) had readily accepted the stain. This was clear also in transverse sections, the thinner blue lines alternating with the white prisms. Toward the depths of the lesions, where the staining was lighter, the stain could be seen between the prisms and penetrating as fine hairlike lines into what appeared to be sound enamel.

In some transverse sections, cracks and lamellae were seen to be stained, but the occurrence of cracks or lamellae could not be linked with the occurrence or apparent configuration of the lesions. In some lesions, the carious process and the lamellae corresponded, but there was no indication of any etiological relationship between the two.

(c) Concerning the appearance of the structural phenomena *the Hunter-Schreger lines* (Fig. 1) appeared to bear little relationship to the main part of the lesions. However, in some specimens where the lesion did not reach the dentinoenamel junction, the apex of the lesion tapered off into fingerlike extensions. Each extension was limited to one Hunter-Schreger line, and they were separated from each other by a distance corresponding to the width of a Hunter-Schreger line. This appearance was not seen in all sections, mainly because many of the lesions extended to the dentinoenamel junction. However, the relationship appeared to be by no means a fortuitous one.

*The striae of Retzius* (Fig. 4) seemed to exercise a strong influence on the shape and appearance of the carious lesions. In longitudinal sections of lesions, little evidence was seen except the occasional relationship between the lateral margin of a lesion and a stria. However, in transverse sections the evidence was much more clear cut. Viewing the specimens by direct incident light, the normal enamel presented a smooth, shiny surface. In this could be seen an area, corresponding to the carious lesion, whose surface was somewhat matt. This area was not structureless, but presented a layered effect, each layer being parallel to the enamel surface. The layered or striated effect could also be seen by dark ground illumination because the striae of Retzius had accepted the dye, and stained a deep blue. In the more lightly stained parts, the stained substance between the enamel prisms could be seen to merge with stained striae. In those parts of the lesion where the carious process began, actual cleavage of the enamel occurred, and this cleavage could be seen to have occurred along the striae of Retzius.

The outline of the lesions in the transverse sections bore a close relationship to the striae of Retzius, the lesions having a flattened internal margin, which in shallower lesions often remained quite parallel to the enamel surface.

2. *Lesions stained with alizarin red S:* Here again, the most obvious macroscopic feature was the fact that the carious lesions were the only portions of the enamel to become stained. The normal enamel remained quite white, while the lesions became a bright cerise color. Any remaining normal dentin or cementum became stained but not quite to the extent that the lesions were stained. On removal from the alizarin red S solution, there was normally a red film covering the entire enamel surface, but this was readily removed with a moist cloth, leaving the stain only in the lesion. This stain resisted removal even by the action of acid.

(a) The lesions had shapes similar to those already described in the paragraphs referring to the teeth stained by methyl green.

(b) A great deal of similarity was found to exist between the appearance of the components of the enamel in this series of specimens and those stained by methyl green. In longitudinal sections, the central portions of the lesion appeared as a light red, rather amorphous mass, although occasionally the outline of the prisms, entirely stained red, could be seen. The deeper parts of the lesion usually showed areas where the red stain was limited to the substance between the prisms. Segmentation could also be seen, the transverse striations appearing red.

In tangential sections the same appearances could be seen. It was noted, however, that in some of the very small lesions the staining of the prisms was almost lacking, the entire field appearing as a series of white, somewhat hexagonal areas surrounded by a fine network of a stained substance.

(c) *Hunter-Schreger lines:* A description has already been given of the lobulated or finger-like projections in some of the early lesions when viewed in longitudinal sections, and their apparent relationship to the Hunter-Schreger lines. This effect could again be seen in this series of specimens.

*Striae of Retzius:* The patterns already described in the previous series were seen again. Here, the striae were stained a deep red and exhibited the same "layering" or striated effect as before. The relationship of the striae to the lesions and their influence on the shape of the lesions were again apparent.

*Conclusions.*—(a) The shape of the early lesions originating on smooth enamel surfaces as seen in longitudinal sections did not differ from that previously described by numerous investigators. The lateral borders of the lesions tended, both occlusally and gingivally, to be determined by the direction of the prisms, and on some occasions they bore a relationship to the striae of Retzius. Thus, a sharp indentation would sometimes occur corresponding to the position of a stria of Retzius. The deeper portions of the lesion often had a definite relationship to the Hunter-Schreger lines, and the lesions could be seen terminating in a series of fingerlike processes which corresponded to the Hunter-Schreger lines. Reference to this has not been

found in the literature, but it can easily be imagined that the arrangement of prisms within the Hunter-Schreger lines would lend itself readily to the production of such a picture.

The appearance of lesions as seen in transverse sections is quite different. Here the conical form of the lesion is absent and the progress of the lesion shows a clear relationship to the occurrence of the striae of Retzius, which appear as concentric rings.

(b) These observations concerning the appearance of the components of the enamel confirm those made over the past years by other investigators. Although the body of the lesion in many cases was uniformly stained, the more lateral and the deeper portions regularly displayed striations in the prisms and, at the advancing margin, the stains could be seen between the apparently normal prisms. It was interesting that both staining methods produced identical pictures. It would appear, then, that penetration by dyes is probably easiest along the organic tissues, and also that calcium salts are in the most readily available form (ionized) in those areas. This interpretation was strongly supported by the appearance (described as "steps and stairs") of the lesions in transverse sections where the penetration of the lesions appeared to be intimately bound up with the distribution of the organic structure within the enamel.

No relationship could be established at any time between the presence of lamellae and lesions. Although lamellae were seen at times in relation to lesions, their presence did not appear to be essential, and there were many lesions in which no lamellae could be seen.

(c) It has been suggested that the most logical interpretation of the Hunter-Schreger lines is that bundles of prisms, bearing little directional relationship to the adjoining prisms, pass as a group from the dentinoenamel junction to the enamel. This concept is quite compatible with these observations, namely, that lesions within the enamel terminate in a series of projections which correspond to the Hunter-Schreger lines.

Although the lateral walls of the lesions as seen in longitudinal sections did at times appear to bear some relationship to the presence of striae of Retzius, this relationship was not observed sufficiently often to be interpreted as more than a casual relationship. However, in transverse sections the lesions bore quite a distinct relationship to the position of striae of Retzius, and the striae themselves, in such positions, were stained by the various dyes. If the concept that striae represent slightly less calcified "pauses" in the development of the enamel is correct, it would seem reasonable to assume that there will be a greater concentration of organic material at such sites. It is suggested that such concentrations determine the progress of the lesion.

### THE EXAMINATION OF LESIONS BY TRANSMITTED LIGHT

After the completion of the work described previously, it was decided to attempt to correlate the appearances in specimens examined by incident light

with those in sections examined by transmitted light. A series of ground sections was therefore prepared, some of which were stained by alizarin red S and some left unstained.

Fig. 6.

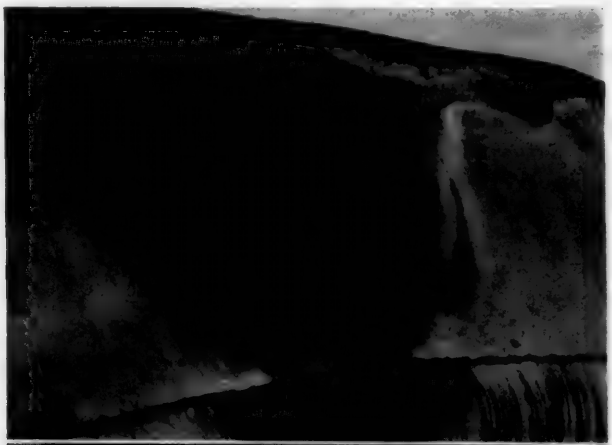

Fig. 7.

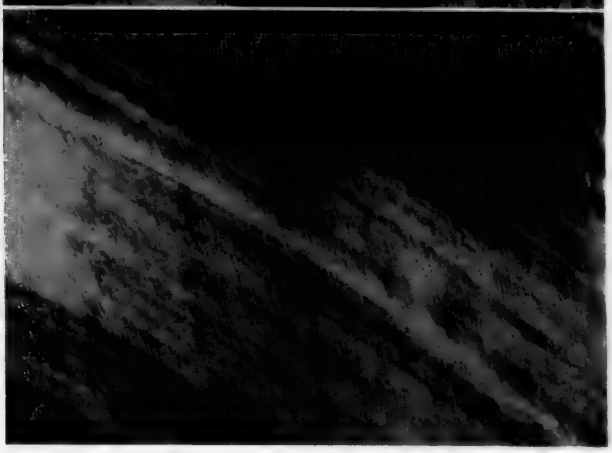

Fig. 6.—Longitudinal section of carious enamel lesion stained with alizarin red S. Relation of base of the lesion to the Hunter-Schreger lines can be well seen. (Magnification, ×65. Tranmitted light.)

Fig. 7.—Base of a lesion as seen in longitudinal section. Relationship of margin of lesion to striae of Retzius is obvious. (Magnification, ×100. Transmitted light.)

It was found extremely difficult to prepare ground sections through the lesions in a transverse plane because the enamel had usually cracked before the sections were considered sufficiently thin for examination. The majority of specimens which were examined were, therefore, longitudinal sections. Again, this study was confined to lesions occurring on the approximal surfaces of teeth.

The examination of longitudinal sections of unstained lesions confirmed, in general, the numerous observations which have been reported by other investigators (Fig. 6). The outline of the lesions was usually determined by the direction of the enamel prisms. Longitudinal sections of the lesions were often cone-shaped, such an appearance being due to a gradual fading out of the carious process both coronally and gingivally. At times, the lateral margins of the lesion were modified by the existence of the striae of Retzius, although this was not common (Fig. 7).

Transverse striations in the affected enamel prisms were commonly present, particularly in the smaller or earlier lesions. The brown coloration of the lesion was often noted, but this was in all probability often due merely to a change in the refractive properties of the disrupted prisms, and not an inherent pigmentation.

Observations on smaller lesions confirmed the relationship between the Hunter-Schreger lines and the deepest prolongations of carious lesions. If a carious lesion is examined by transmitted light and by incident light, the Hunter-Schreger lines and the fingerlike projections at the apex of the lesion are seen to coincide accurately.

It was noted again that carious enamel stained readily with alizarin red S and that the normal enamel did not. The entire lesion stained uniformly and had an amorphous appearance. However, in the ground sections it was difficult to determine satisfactorily the regions in which this stain is first accepted.

## DISCUSSION

Because enamel is very highly calcified, it would seem impossible to obtain a complete picture of the conditions within carious lesions by the examination of ground sections alone. So many artefacts can develop and the phenomena observed can be interpreted in such a variety of ways that the conclusions reached will almost certainly be biased by the investigators' previous ideas concerning the structure of enamel and possible methods of its breakdown. Many interesting observations can be made, however, and ground sections have been extensively used in the study of this problem.

The first noteworthy fact which has been confirmed by this work is that whereas normal enamel under the conditions of these experiments remained unaffected by the dyes used, the carious lesions accepted the dyes with great avidity. This means at least that the enamel structure has been so changed that the carious areas have become more permeable to, or have developed an affinity for, such chemicals. The use of methyl green gave no hint as to the nature of the tissue component involved, but adequately demonstrated the change. Alizarin red S solutions, however, revealed other interesting properties. It is known that a very obvious red lake is formed when this dye is in contact with calcium salts. However, it would appear that in the structure of normal enamel, the calcium is so bound that it is incapable of reacting with the dye. It was assumed, therefore, that the formation of red lakes in carious lesions indicated that in those particular regions the calcium was now in a more

available, or even a free, form. The most obvious deduction is that the lakes were forming in areas where at least partial decalcification or disorganization of chemical structure had taken place, particularly as steps were taken to see that the development of color was not due merely to a change in pH of the solution.

The second point worthy of note is that in carious lesions the organic structures of the enamel and the "free" calcium ions seem to coexist in the same places. Such a condition could mean that the protein structures had undergone degeneration and so allowed the dyes to penetrate along those pathways more easily, thus allowing any calcium salts normally present in their structure to attract alizarin red S to them. It could also be interpreted as indicating that there had been a disorganization or partial removal of calcium salts from the protein material, thus facilitating the passage of the dyes along the (still normal) protein and making any remaining calcium salts more available for interaction with the alizarin red S. When both of these states are normal (that is, normal protein in its normal state of calcification), it is impossible for dyestuffs to penetrate the organic structures, under the conditions of the experiments.

The correlation of the appearances of lesions as viewed in longitudinal and transverse section would indicate that the organic constituents of the enamel play a great part in determining the shape of the lesion. Therefore, it would seem that the protein constituents are either acting as barriers to the extension of the lesion or that they are the pathways along which the carious processes most easily spread. The work carried out with ground sections did not assist at all in determining whether such spread is due to the removal of calcium salts or to the degeneration of the protein constituents or to any other phenomenon, for example, the breaking of a loose union between protein and calcium salts.

## SUMMARY

1. Early lesions of enamel, some stained with methyl green and some with alizarin red S, were examined by incident and transmitted light.

2. Normal enamel remains uncolored by these dyes, but carious enamel stains quite readily.

3. The lesions appeared conoidal in longitudinal sections and rectangular in transverse sections, thus possessing a shape similar to that of a wedge.

4. The deepest parts of the lesions bear a relationship to the Hunter-Schreger lines; the striae of Retzius have a strong influence on the general shape of the lesion.

5. In the deepest parts of the lesions, segmentation of the individual prisms can be seen, and the earliest sign of the carious process appears to be limited to the substance between the prisms.

6. The sites in which organic structures are known to occur seem to be those most intimately linked with the carious processes. Decalcification of these areas seems likely to have occurred, but there is no indication as to whether the organic material has been affected or not.

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# THE FORMATION OF EARLY CARIOUS LESIONS IN DENTAL ENAMEL

## PART II

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## INTRODUCTION

IN a previous paper,[1] I described the appearance of early carious lesions, relating their shape and progress to the different components of the enamel.

The next step was to seek in vitro evidence which might assist in deciding which of the possible interpretations of these previous observations were correct. A series of experiments was therefore undertaken in which various substances were applied to normal enamel and any resulting "lesions" stained and examined. The pathways by which the staining reagents penetrated the enamel were also observed. An attempt was then made to relate these observations to those made on naturally occurring lesions.

## EXPERIMENTAL

### EFFECT OF BUFFER SOLUTIONS ON NORMAL ENAMEL

It would seem that only two main processes are involved in carious lesions in enamel, namely, a disorganization of the calcified tissues and of the organic constituents. Probably at some stage both processes go on, but it is not known which of them is the more important. The first series of experiments was, therefore, concerned with the results of applying substances which could bring about decalcification.

Following the experiments of Enright, Friesell, and Trescher,[2] a buffer solution (pH 4.5) saturated with calcium orthophosphate was prepared, and the enamel was exposed to its action intermittently for short periods, rather than continuously. By this means it was hoped that any decalcification would be slower and possibly more in the manner which, we assume, is characteristic of in vivo caries.

*Materials and Methods.*—

1. *Selection of areas of enamel:* Freshly extracted teeth of all types were washed and dipped into hydrogen peroxide to remove the debris. The teeth were then dried and examined by means of a dissecting microscope and hand lens. Areas (roughly 2 by 3 mm.) on approximal surfaces which appeared to be entirely free from any lesion or other physical defect were demarcated by means of a pencil line.

2. *Preparation of the enamel:* The teeth were mounted upon wooden applicators (thin rods 6 by $\frac{1}{16}$ inches) by enlarging the root canal with a bur

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and forcing a sharpened applicator into it. Each tooth was then placed in a test tube with the applicator protruding through and beyond the wool plug and autoclaved at fifteen-pound pressure for ten minutes. This method of sterilization was chosen as least likely to interfere with or to alter the enamel structure. After autoclaving, the junction of the root of the tooth and the applicator was carefully and aseptically cemented with sticky wax. The whole tooth was then dipped into sterile, molten paraffin wax. After the thin coating of paraffin wax had completely set, each tooth was removed in turn from its test tube and the area of enamel previously outlined in pencil was freed of its wax covering by means of a sterile scalpel, thus producing "windows" in the paraffin coating and exposing the chosen areas. After the windows had been prepared, each tooth was immersed in a tube of sterile nutrient broth and incubated at 37° C. to check the sterility of the preparation. If growth occurred in the broth, the tooth was discarded.

3. *Preparation of solutions and media:* (a) Broth: In all experiments, simple nutrient broth, to which 0.1 per cent gelatin had been added, was used. (b) Buffer solution: A simple buffer solution was made by adding sufficient 0.2 N sodium hydroxide to 0.2 N lactic acid to give a hydrogen ion concentration corresponding to a pH of 4.5. Finely ground calcium orthophosphate was then added to the solution in excess and the mixture shaken at intervals for three days, when the supernatant fluid was filtered off and the pH restored to 4.5 by the addition of 0.2 N lactic acid. This solution was sterilized by Seitz filtration.

4. *Submission of the enamel to the action of reagents:* The teeth were divided into two groups, namely, those to be treated with a buffer solution of pH 4.5 and those to serve as controls. The teeth in the first group were placed in separate tubes containing sufficient buffer solution to cover them. They were allowed to remain in the buffer for eight hours and were then removed, washed in sterile broth, and placed in an empty sterile tube for sixteen hours, when they were returned to the buffer solution and the process was repeated. The teeth in the control group were placed in nutrient broth and left undisturbed.

5. *Length of the experiment:* Some teeth from each group were removed for examination at the end of each month and the experiment was terminated at the end of three months.

6. *Variations in the experimental conditions:* After some weeks the pH of the buffer solution was adjusted to 4.1 to avoid the development of fungal contaminants. During the last month of the experiment, the teeth were allowed to remain in the buffer solution for twenty-four hours instead of eight hours of each day.

7. *Examination of the specimens:* As each tooth was withdrawn from the experiment, it was washed and stripped of the paraffin and sticky wax, with due care taken to avoid removing the pencilled outline on the enamel. All teeth were then examined with a hand lens and under a dissecting microscope, after which the roots were removed and the crowns prepared for microscopic examination.

The majority were then stained with alizarin red S, and ground and polished sections prepared in different planes. Some were prepared by embedding the enamel so that the external surface was protected and the dentinoenamel junction lay uppermost toward the exposed surface. The specimens were then ground until the first signs of staining were observed, thus exposing the deepest portion of the affected tissues.

*Results.*—Before describing the results, it should be reiterated that the object of these experiments was not to produce carious lesions in vitro but to examine the effect of selected reagents on normal enamel and to determine which structures were earliest affected.

(a) Concerning the effect of the buffer solutions, pH 4.1 – 4.5 (Figs. 1 through 3), the teeth treated with the acid buffer solution for the shortest time showed practically no effect. These teeth, after staining with alizarin red S, occasionally developed a very light pink color in the experimental area, but the most careful examination revealed only a homogeneous coloration over the entire surface.

Those teeth treated longer with the buffer solution showed some degree of dullness and opacity, such as is commonly seen after decalcification. The surface remained quite hard, however, and there was no apparent loss of surface continuity. In many of the teeth, quite a considerable amount of the normal sheen still remained over these areas, and a cross section through the treated areas showed extremely little change in the subsurface structure, indeed in many cases the change was practically restricted to the surface of the enamel.

On staining these areas with alizarin red S, a bright red color developed over the entire area. Microscopic examination revealed that the entire surface of the enamel had been affected, that is, both the exposed ends of the prisms and the substances between the prisms had uniformly accepted the stain. The prism ends tended to appear somewhat concave, but it could not be decided whether more destruction had occurred in the body of the prism, or in the interprismatic substance. In cross section, the staining appeared throughout the depth of the "lesion" in a practically homogeneous fashion.

The examination of sections prepared by grinding from the dentinoenamel junction toward the experimental surface revealed a very different picture. Using the highest magnifications, it was found that the first signs of red coloration appeared in the prism sheaths. As more enamel was ground away, the red areas became thickened, as though the interprismatic substance were being involved. After further grinding, that is, in the more superficial areas of the "lesion," the rods themselves were seen to become involved, until the entire area was colored red.

It was not possible in the longitudinal preparations to observe any cross-striation of the prisms, in part because the depth of the penetration did not allow a great area to be examined. Likewise, it was impossible to note the effect of these buffer solutions on the Hunter-Schreger lines or striae of Retzius.

Fig. 1. Fig. 2. Fig. 3.

Fig. 1.—Tangential section of enamel which has been subjected to the action of acid and then stained with alizarin red S. The red stain can be seen in the areas of the prism sheaths. (Magnification, ×1,000. Incident light.)

Fig. 2.—Same preparation seen in Fig. 1 but now ground to a level closer to external surface. Thickening of the area of stain around prisms can be seen. (Magnification ×1,000. Incident light.)

Fig. 3.—Same preparation seen in Fig. 1 but now ground to a level still closer to the external surface. Complete staining of rods can now be seen. (Magnification, ×1,000. Incident light.)

(b) Concerning the appearance of the control specimens, no changes could be determined in the enamel of the teeth in the control group after examination by naked eye and microscope using both unstained sections and sections subjected to alizarin red S.

*Conclusions.*—The main conclusions drawn from observing the effect of acid on normal enamel under the experimental conditions imposed were, first, that the enamel became more permeable to the penetration of dye substance and, second, that the staining appeared to progress along the prism sheaths, spreading to the interprismatic substance and rod substance. This is virtually what is seen in early carious lesions. The effect of the acid on the enamel appeared to be one of decalcification although it must be stressed that one could not dismiss the possibility of organic substances being involved.

### HISTOCHEMICAL REACTIONS IN NORMAL AND CARIOUS ENAMEL

*A. The Ninhydrin Reaction.*—This reaction[3, 4] depends on the action of an aqueous solution of triketohydrindene hydrate (ninhydrin) on organic material in a neutral solution. Common procedure is to immerse the tissue to be tested in a mixture of a 0.4 per cent solution of ninhydrin and a phosphate buffer of pH 6.98. The container is then placed in a boiling water bath for one to two minutes. The development of a blue or violet color indicates the presence of amino acids, free or bound in peptides or proteins. This test is believed to be specific for all amino acids except proline and hydroxyproline, for peptides, and for some other compounds, such as aldehydes. However, the reaction is far less sensitive and the color much more reddish with compounds other than amino acids. The possibility of confusion by their presence can usually be readily eliminated. This conclusion on the validity of the interpretation of color development was confirmed by observation on the reaction with different substances, such as hair, collagen, peptone, and gelatin. Only when degradation of the protein had occurred was the classical color obtained.

Using the technic described by Serra,[4] tests were carried out first on normal enamel and dentin. It was found that when dentin was present a blue color developed rapidly within the solution and the dentin. However, solution in contact with enamel only did not change color on any occasion, and careful microscopic examination of the enamel did not reveal any blue color.

Specimens of pure enamel (that is, free of any debris, lesions, or dentin) were then placed in small centrifuge tubes and dissolved by means of hydrochloric acid. The solution was spun and the supernatant fluid removed and stored. The extremely scanty deposit was resuspended in distilled water and then recentrifuged, and the supernatant fluid again removed. Ninhydrin reagent and buffer solution were added to the deposit, and this was then placed in the boiling water bath. The supernatant fluids and washings were also tested for ninhydrin reaction after neutralization. None of these tests gave a color reaction.

The test described previously was carried out several times, always with the same result. On several occasions, larger quantities of enamel were used, and after the residual, decalcified organic matrix had been subjected to the action of the ninhydrin reagent, it was transferred to a welled slide and examined carefully for evidence of coloration. It was then subjected to Millon's reagent, and it was found that the material became a bright red, indicating that the material was protein in nature. These observations were repeated some twenty times.

A large number of pieces of pure enamel were submitted to the action of a weak acid solution for about forty-eight hours. They were then checked for the presence of any impurities in the form of residual dentin or organic debris, washed, and placed in a 10 per cent celloidin solution, which was drained off after some twenty-four hours and replaced by 70 per cent alcohol for twenty-four hours. By this means, the separate pieces of enamel were coated with a thin sheath of hardened celloidin. These specimens were then submitted to the action of either 5 per cent trichloracetic acid or 3 per cent hydrochloric acid until decalcification appeared to be complete. They were next suspended in 95 per cent alcohol in individual tubes and washed twice with an alcohol-ether (equal parts) solution, the solution being carefully removed by suction. The free, decalcified protein was then suspended in water. Some of the samples of decalcified protein were then immersed in a few drops of chemically pure hydrochloric acid, the tube sealed, and placed in a boiling water bath until the protein was completely digested. This digest was neutralized with concentrated sodium hydroxide. All samples were then subjected to the ninhydrin reaction. It was found that the samples of decalcified protein still failed to react but that the samples of protein which had been digested in the boiling acid gave a strong reaction.

Some twenty-five specimens of enamel in which carious lesions were present were prepared by cleansing thoroughly and gently polishing the external surface of the lesion with a fine dental disc in order to remove any surface debris. Specimens were then subjected to the action of the ninhydrin reagent, the effect of which varied somewhat. On some occasions the supernatant fluid changed to a faint blue color and on others it remained colorless. Within the lesions there was at times a slight suggestion of a blue coloration, but examination of the lesions under high magnifications did not confirm its presence.

Pieces of enamel were then taken in which lesions were present. The enamel surrounding the lesion was reduced to a minimum by means of diamond discs and the lesions were then decalcified with 3 per cent hydrochloric acid. After decalcification, the residue was submitted to the ninhydrin reagent and carefully examined. It was found that some of the organic matter stained quite a deep blue to purple, and some did not.

*Conclusions.*—The absence of ninhydrin reactions in the supernatant fluids and the decalcified organic matter would indicate that the protein

structures within the normal enamel are not destroyed by acid decalcification. The reaction given by digested protein indicates that it is only after the most rigorous attack that protein structure of the enamel can be broken down by acids.

The ninhydrin reactions given by some of the decalcified organic matter of carious lesions cannot be readily explained, but the possibility of penetration of organic debris, or bacteria, from the saliva into the lesion during its formation deserves serious consideration.[5]

*B. The Morgan-Elson Method.*—The Morgan-Elson test is of use primarily for indicating the presence of glucosamines. Glucosamine and chondrosamine react with acetyl acetone in a boiling alkaline solution to give a pyrrole which condenses with Ehrlich's reagent (para-dimethyl-amino-benzaldehyde dissolved in acid alcohol) giving a purple color. The material to be tested is placed in an alkaline solution of acetyl acetone, which is then heated in a boiling water bath for fifteen minutes; after cooling, alcohol is added, followed by Ehrlich's reagent. There is a slow evolution of carbon dioxide, and the presence of glucosamines is indicated by the development of a purple color. In solution, this test can be carried out quantitatively as a colorimetric estimation.

Material was prepared from carious enamel and subjected to the test using glucosamine as a control. Although the results were not clear cut, all material tested appeared to give a positive reaction. It was obvious, then, that before any conclusions could be drawn, normal enamel had to be tested.

Numerous pieces of enamel were then taken and carefully checked to ensure the absence of dentin or surface impurity. They were decalcified by the celloidin method previously described. After decalcification, some specimens were dissolved by boiling in a few drops of a concentrated acid. The samples of both the decalcified and the digested protein were then subjected to the Morgan-Elson reaction. It was found that the decalcified protein gave a strong reaction; indeed, it was stronger than the reaction obtained with the digested protein, which was only weak.

It was noted also that when testing whole sections of teeth, the lamellae and tufts in normal enamel were colored quite a bright red.

*Conclusions.*—It was concluded from these results that the organic structure contains a hexosamine, which indicates the existence of a mucopolysaccharide. The strong reaction obtained after decalcification may well confirm the work of Sognnaes and Wislocki,[6] who described the rapid depolymerization of the acid mucopolysaccharide in enamel during the earliest stages of the formation of a lesion.

*C. The Hotchkiss Method.*—Engel[7] reported that fully differentiated dentin did not react with the periodic acid-leucofuchsin reagent of Hotchkiss.[8] On the other hand, he claimed that carious dentin stained a brilliant red. As this observation was based on the depolymerization of the carbo-

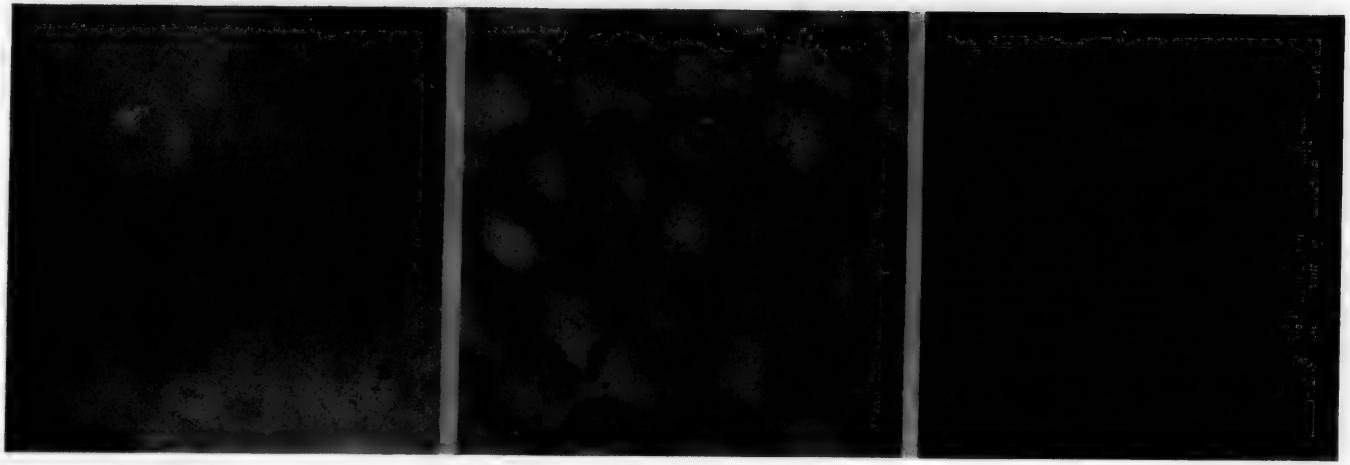

Fig. 4. Fig. 5. Fig. 6.

Fig. 4.—Tangential section of normal enamel which has been immersed in silver nitrate. Staining of the prism sheaths can be seen. (Magnification, ×1,500. Incident light.)

Fig. 5.—Same section of enamel seen in Fig. 4 but now ground to a level closer to external surface. Apparent penetration of interprismatic substance and rods can be seen. (Magnification, ×1,500. Incident light.)

Fig. 6.—Same section of enamel seen in Fig. 4 ground still closer to external surface. Involvement of all structures can be seen. (Magnification, ×1,500. Incident light.)

hydrate-protein complexes, it was decided to attempt to apply this test also to normal and carious enamel. The test consists of subjecting the specimens to a solution of periodic acid followed by a reducing rinse. The material is then placed in fuchsin sulphite, washed with sulphite water, and dehydrated.

Attempts to apply this test to normal and carious enamel were not successful, nor could Engel's observations be confirmed.

*D. The Silver Nitrate Method.*—The change wrought in enamel by the application of acid is probably due to decalcification, but the possibility that the organic material is changed has not so far been eliminated. Evidence in support of this view was sought by examining the paths by which normal enamel is penetrated by fluids incapable of decalcifying it.

In this regard, the value of silver nitrate solutions has long been known. Teeth with areas of apparently normal enamel were placed in an aqueous solution of silver nitrate for several hours. They were then washed, exposed to light, and prepared by transverse section for examination by incident light. Likewise, teeth with early carious lesions in the enamel were subjected to similar treatment. Some of the specimens were so prepared that the external surface of the enamel could be examined, while others were so embedded that they could be ground and polished from the dentinoenamel junction toward the tooth surface until the first signs of silver nitrate penetration were observed. In this manner, both the surface and the greatest depth of penetration in cross sections could be examined.

*Results* (Figs. 4 through 6).—In normal enamel, penetration was found to occur with comparative ease, and the dark brown stain of the reduced silver nitrate could be readily detected. On the external surface of the enamel, the stain seemed to be quite generalized, and no particular structure of the enamel was affected more than another. However, when the deeper parts of the enamel were examined, the stain was found to be limited entirely to the prism sheaths. By successive grindings of the section, first the areas adjacent to the prism sheaths and finally the entire substance of the section could be seen to have been penetrated.

In carious enamel, a picture very similar to that already described was seen. Transverse sections of lesions again exhibited the striations and steps-and-stairs outline; features related to the existence of striae of Retzius. As before, the main part of the lesion tended to have an amorphous appearance but, in the deeper parts, penetration could be seen proceeding along the lines of the prism sheaths.

*Conclusions.*—The effects of silver nitrate as a staining reagent would indicate, first, that normal enamel is permeable to molecules of small dimension and, second, that such permeation can occur most readily along organic pathways of the enamel. In carious enamel, this permeability is vastly increased, but the penetration apparently still continues along organic pathways. It is reasonable to assume that the silver nitrate solution cannot bring about the breakdown of the protein. Therefore, it might be argued that it

is not necessary (although not so proved) for the organic framework of the enamel to be broken down in order to get the pattern of penetration seen in carious lesions.

## Discussion

It is pertinent at this juncture to refer to similar work of other investigators and to see what interpretation they placed on their observations. Pickerill,[9] discussing the physical properties of the enamel surface, pointed out how perfectly sound teeth, when placed in a 10 per cent solution of silver nitrate, could quite readily be penetrated by this substance. His main interest at this stage was in the permeability of the enamel, but he remarked that "it is perfectly obvious that all that is stained by the nitrate of silver is not organic matter, since the prisms themselves are stained." Later, he used the phrase "showing the interprismatic substance stained, but not the prisms themselves." It should be noted that the photomicrograph referred to was a low-power magnification, and it is suggested that had Pickerill examined his sections under higher magnification, he would have noted that the stain was localized, not in the interprismatic substance, but in the prism sheaths. The results obtained by us would then be identical with those obtained by Pickerill. To quote Gottlieb:[10] "Figure 61 is a ground section of a beginning enamel caries, and it shows exactly the same picture as the ones seen with the silver nitrate impregnation. The peripheral part of the enamel is dark brownish in the ground section. . . . The histological picture of dental caries appears duplicated by silver nitrate in all details. The organic parts of the enamel are affected first. This means, caries is a proteolytic process spreading along and affecting the organic parts. It has not the slightest similarity to the result of acid action. . . . However, if the acid component is present without the yellow pigment, caries does not develop and only chalky enamel occurs. Both components have this in common, that they invade the tooth structure through the media of the organic matter of the lamellae or the uncalcified prism sheaths." It can be seen again that our observations on normal and carious enamel are similar to those of Gottlieb, but our interpretations differ widely. It is agreed that the paths of penetration of enamel by silver nitrate and by the carious process apparently are similar, and also that the pathways by which the first penetration is made are apparently the prism sheaths. However, there is nothing in our observations which would indicate that the protein undergoes the destruction which Gottlieb suggests. Admittedly, no evidence has been adduced by us to indicate that some breakdown does not occur, but the observations certainly indicate that if acid is the attacking agent, the protein sheath can be hydrolyzed only by attack of such intensity that it could not occur within the mouth. Further, Gottlieb offers only histologic evidence for the degradation of the protein moiety.

It can be seen that the minutiae observed in carious enamel by a number of workers are basically the same, even though various interpretations have been placed on their work by different investigators. The observations of Frisbie and Nuckolls[11] are also similar, but they, too, differ from other workers in their interpretation of them. It would seem, then, that once certain basic facts have been established by observing ground and decalcified specimens, we must exploit histochemical technics with a view to determining the fundamental chemical processes within the carious lesion.

The use of alizarin red S and the study of ground sections indicate clearly that demineralization occurs in all carious lesions, an observation in accord with those of most authors.[6] The removal of calcium is initially effected within the prism sheaths, and penetration occurs along these organic pathways. If the terms "prism sheaths" and "interprismatic substance" were clearly defined, it would be easier to say whether this latter observation were in accordance with that of others or whether some observers believed the interprismatic substance to be primarily affected.

Whether any breakdown of the organic substance occurs is a point which must be decided by the use of histochemical methods. Frisbie and Nuckolls[11] have claimed that proteolysis is the major factor, and they base their observations on decalcified sections. Gottlieb[10] also claimed that proteolysis is of major importance, basing his claims likewise on the observations of ground sections. It would seem that neither of these claims is sufficiently substantiated, and before any decision can be made, both normal and carious enamel must be examined in detail by histochemical means. Such methods have only recently been employed to study carious enamel. Pincus[12] reported that he found enamel protein to be a mucoprotein capable of resisting hydrolysis.

Hutton and Nuckolls[13] have reported that enamel protein did not undergo immediate extensive hydrolysis at room temperature. Indeed, it would appear from their results that it was not until enamel protein was treated with 20 per cent hydrochloric acid for two hours at 120° C. that they were able to observe extensive hydrolysis. In order to obtain these results, they used paper chromatography and ninhydrin spot tests. These findings agree with our observations.

Wislocki and Sognnaes[14] used various histochemical technics in an attempt to demonstrate the presence of different substances and conditions in the enamel. They found that ground sections of fully mature, normal enamel were practically unaffected by the stains employed. They then fixed them in Zenker's fluid in preparation for basophilic staining. This brought about sufficient decalcification of the enamel to permit access of the stain to its organic framework. The results indicated the presence of an acid mucopolysaccharide within the organic framework of the enamel. Sognnaes and Wislocki,[6] using various staining technics and also certain histochemical methods, which included the periodic acid-Schiff reaction and also tests for

alkaline phosphatase, sulfhydryl groups, and keratin, reported that caries of the enamel was initiated by the existence of a deficient mineralization or the partial demineralization of "the organic elements between the enamel prisms," followed by a "depolymerization of the acid muco-polysaccharide which surrounds the enamel prisms." They believed this depolymerization to be an enzymic one. They have interpreted the disappearance of metachromatic and basophilic reactions in carious lesions as indicative of the disappearance or degeneration of acid mucopolysaccharides. It might be well to note, however, that both acid solutions and silver nitrate solutions were capable of penetration into the rods themselves after they had penetrated along the organic pathways. It is doubtful whether the silver nitrate could bring about a depolymerization of any mucopolysaccharide. Therefore, in carious lesions it would not seem to be essential for the acid mucopolysaccharide to be depolymerized prior to the invasion of the prisms. However, our results, in which a strong positive Morgan-Elson reaction was obtained after decalcification of normal enamel, lend weight to their conclusions.

Reviewing the various observations, it is clear that, first, normal enamel is impermeable to most substances. If, however, we use substances composed of small molecules, for example, silver nitrate, we find that they are able to penetrate into the enamel. It will be seen that such substances penetrate by a certain fixed route, namely, along the lines of distribution of organic material within the enamel. There is no suggestion of a general diffusion into the other parts until quite a considerable amount of penetration along these paths has occurred.

When we subject enamel to the action of acid instead of solutions such as silver nitrate, the acid (whose action is revealed by staining with alizarin red S) proceeds along identically the same lines as the silver nitrate.

If we now study the mode of extension of early carious lesions, we find that it too follows paths similar to those described in relation to silver nitrate and acids. It will be seen, by staining with alizarin red S, that one possible mechanism of their formation is that of demineralization. Our interpretation is that the carious process penetrates first along the organic sheaths of the prisms and then spreads laterally into the interprismatic substance and into the rods themselves until a somewhat decalcified, homogeneously staining mass remains. The outline of the lesion is further controlled by the presence of more substantial organic structures, such as the striae of Retzius, and at the limits of lesions, the earliest phase of the process may be seen confined to one group of prisms representing one particular Schreger line.

There is reliable evidence that this demineralization is the first step, and therefore the most important step, in the formation of carious lesions, an observation in keeping with the views of the majority of investigators. However, reasons must be sought to explain why demineralization should proceed along such definite pathways. Three possibilities suggest themselves: first, that a more soluble form of the inorganic salts exists in these areas; second,

that the organic material within the enamel acts as a channel (or that there is, in addition, a loosely bound calcium proteinate) from which the inorganic salts can be removed more easily, and thus the lesion can progress more rapidly along those paths; third, that the protein material is destroyed by some form of proteolysis at an early stage of the development of the lesion, thus making it possible for acid to pass along the pathways which originally were filled with protein material. We were unable by any means to adduce evidence that the protein within the enamel was destroyed at some particular stage of the carious process. We were, however, able to show that the protein was highly resistant to acid except under conditions which could not occur in the mouth.

## SUMMARY

1. The permeability of enamel subjected to the action of acid buffer solution was noticeably increased. When stained with alizarin red S on the surface, all structures were uniformly involved. At a deeper level, the affected areas were restricted to the margins of the prisms, the prism sheaths, and interprismatic substance, while the deepest penetration was confined to the areas of the prism sheaths.

2. Ninhydrin, Morgan-Elson, and Hotchkiss tests were made on enamel under various conditions. These revealed that (a) the protein structure of enamel is not broken down by acid decalcification; it can be broken down by boiling in strong acid; and (b) the protein of enamel contains a hexosamine indicating the existence of a mucopolysaccharide.

3. Enamel is permeable to solutions of silver nitrate. The route by which such solutions penetrate is identical with that taken by the solutions of lactic acid.

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# IMPORTANCE OF TYPE OF DIET DURING THE SUCKLING PERIOD ON THE INCIDENCE OF EXPERIMENTAL DENTAL CARIES IN RATS

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IN EARLY studies of rat caries in this laboratory,[1, 2] two different strains of animals were used. One was the McCollum strain, which was raised in this laboratory and had available from birth a stock diet containing a large percentage of coarsely ground corn.[1] The other was the Sprague-Dawley strain, which was secured from a commercial breeder.* The latter animals' diet consisted of a finely ground, low-cariogenic commercial stock diet available from birth. When, however, both strains were raised on their respective stock diets for 30 days, after which they were placed on the same diet for 140 days, the McCollum strain was shown to have a higher incidence of dental decay than that secured from the commercial breeder. Originally, it was our impression that the differences in the decay rate of the animals might represent a strain difference, since evidence is available to indicate that hereditary factors[4-8] as well as strain characteristics[9-12] are important when considering the experimental decay pattern in the rat. Therefore, it was of some importance to determine whether this was truly a strain difference or whether environmental factors manifested through differences in the cariogenicity of the diet might account for some, if not all, of the variability of the caries incidence.

It has been reported that the incidence of dental caries in rats[13, 15] and in hamsters[14] is influenced more by the diet prior to the eruption of the teeth than it is following their eruption. Sognnaes[13] found that if rats were kept on a finely ground stock diet (containing, incidentally, 21 ppm of fluorine) until the teeth had completely erupted, caries did not occur on a diet high in sucrose during a period of seven months. However, when the animals were conceived as well as raised on the high-sucrose diet, caries developed in a few months. These findings suggested to him that a purified ration which is high in sucrose is extremely conducive to caries, provided its ingestion is begun before and continued during tooth development.

The period of calcification and the time of eruption into the oral cavity of the rat dentition have been reported.[16] When the rat is about 7 days of age the maxillary and mandibular molars appear in the oral cavity, while the maxillary and mandibular second molars erupt at about 13 days. The rat will ordinarily begin to eat and drink from the supplements provided at about 12 to 14 days, so that if any real protection by anticaries agents is afforded these animals by the food supplements, it may not be indicated in the first and second molars, providing the mechanism of caries reduction depends on a reaction with the calcifying tooth substance and the anticaries agent. Fluorides have

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*Carl Wilson.

been suggested to be such anticarious agents. Before the rat is 12 to 14 days of age, fluorides received by it must come from the mother's milk. Since little fluorine is found in milk under ordinary dietary conditions, it seems improbable that a quantity sufficient to reduce the incidence of decay in rats is available by this route. However, after about 12 to 14 days, the animals may consume considerable amounts of fluorine in the food and water. If this source of fluorine is beneficial in caries prevention, it should be possible to evaluate the effectiveness of preweaning supplements of fluorine by starting such supplements from the twelfth to the fifteenth days of age and making a comparison with a control group receiving similar supplements but beginning at a later age.

It is difficult to simulate, in rats, a condition analogous to that existing in human beings pertaining to the systemic protection of the dentition by fluorine. However, in order to produce a somewhat similar condition, a number of rats which had been previously exposed to caries-reducing agents (fluorides) from 30 to 140 days were bred in this laboratory. Their progeny (second-generation rats) were raised under conditions identical to those of the parents except that they had the fluoride supplements and experimental diet available to them from birth. The purpose of this study was to determine, then, what effect fluorides at low concentrations have in the reduction of experimentally produced decay in second-generation rats whose parents previously had contact with fluorides at a fluoride level sufficiently high to reduce satisfactorily the incidence of tooth decay in the parents. Also, a portion of this paper deals with the investigation of the difference in the incidence of decay in two different strains of rats. As the two strains were raised under different conditions before 30 days of age, the first objective was to control each strain in relation to the type of diet eaten, supplements received, and so forth, until such time as both groups were placed on the experimental diet and supplements.

TABLE I. NUMBER OF FIRST-GENERATION ANIMALS (COLUMNS 2 AND 3) USED TO BREED THE SECOND GENERATION (CARL WILSON STRAIN ANIMALS)

| | FIRST GENERATION | | SECOND GENERATION | | |
|---|---|---|---|---|---|
| GROUP | MALE | FEMALE | TOTAL NUMBER OF RATS BORN | TOTAL SURVIVING | AVERAGE NUMBER OF SURVIVING RATS PER LITTER |
| $SnCl_2$ (Sn, 10 ppm) | 4 | 4 | 97 | 89 | 8.0 |
| $SnF_2$ (F, 8 ppm) | 10 | 10 | 228 | 211 | 7.6 |
| NaF (F, 10 ppm) | 3 | 3 | 59 | 50 | 6.5 |
| $H_2O$ (F, 0.1 ppm) | 6 | 6 | 128 | 103 | 7.1 |

## METHODS AND PROCEDURES

Initially, a large number of Carl Wilson animals were placed on the coarsely ground corn diet and fluoride supplements[1] when 30 days old. After 140 days, forty-six animals, from four different experimental groups, were bred,

and the resulting second-generation animals used for study in this experiment. The remaining animals were sacrificed and the data reported.[1] The forty-six animals saved for breeding purposes were divided as follows: From the first-generation stannous chloride animals (10 ppm of tin), four males and four females were saved, three males and three females from the sodium fluoride group (10 ppm of fluorine), ten males and ten females from the tin fluoride group (10 ppm of fluorine), and six males and six females from the control group (no supplement). Each female was mated to each of the three males in her group during the course of the study. Table I indicates the number

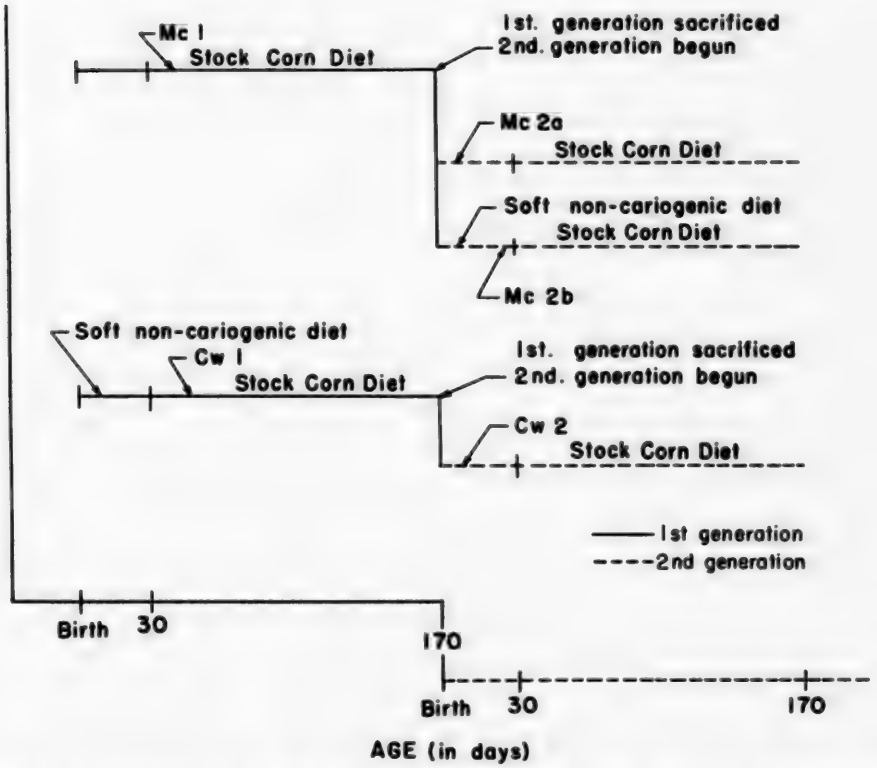


Fig. 1.—A comparison of the diets received by the first- and second-generation McCollum and Carl Wilson strain rats at various ages.

of litters from each group, the mortality rate, and the average offspring from the respective groups. After birth, both the mothers and the young rats were maintained on the identical supplements for 170 days that the parents ($CW_1$) received for the previous 140 days. Thus, for the first 30 days these animals ($CW_2$) were exposed to the caries-producing diet, in contrast to their parents ($CW_1$), which had received our caries-producing diet for the first time at 30 days of age. Another strain, the McCollum strain, which previously had shown a high caries incidence, was used for comparative purposes. A portion of the

McCollum animals were saved for breeding purposes, and their progeny continued as second generation ($MC_{2a}$), while the majority of the group were sacrificed[1] after being on the diet for 170 days ($MC_1$). In order to have a comparable group to the Carl Wilson strain, some of the McCollum strain was placed on a noncaries-producing diet* for the first 30 days of life and then placed on the caries-producing diet for an additional 140 days ($MC_{2b}$). A diagrammatic sketch of the age of the animal on the experimental diets and the diet consumed by the second-generation animals is seen in Fig. 1. The procedure for preparing the heads for examination and recording the lesions has been reported previously.[1, 2]

## RESULTS AND DISCUSSION

Table II shows the reduction in dental caries in the progeny of rats receiving low concentrations of anticaries agents whose parents received the same anticariogenic agents for the previous 140 days. The caries status of the parents at the same age[1] is shown for comparative purposes. The table indicates a marked reduction of decay in the second-generation experimental animals receiving 10 ppm of fluorine as stannous fluoride, but little reduction when sodium fluoride was fed at the same fluorine level. However, this strain (Carl Wilson) had a much greater incidence of decay in the second-generation animals in all groups studied than in the animals of the first generation.[1] Thus, the expected result of increasing the protectiveness against decay in the second-generation animals by furnishing the fluoride supplement twelve to fifteen days earlier did not occur; in fact, the decay pattern appeared to be considerably increased. This led to the speculation that perhaps placing the second-generation rats on the caries-producing diet from birth was more destructive to the teeth than the fluoride supplements were protective. Moreover, the caries pattern of the control animals considerably increased also; so it became necessary to investigate the effect of the cariogenic diet on the caries pattern of the two strains under controlled conditions. The data presented in Table III demonstrate that in the control Carl Wilson animals ($CW_2$) there are 8.8 cavities per rat (sexes combined) in the second-generation animals when they receive the stock corn diet from birth. Thus, under these conditions the incidence of decay in the second-generation Carl Wilson animals ($CW_2$) is similar to that of the McCollum strain ($MC_1$ or $MC_{2a}$). The reason for this alteration in caries pattern may be related to the fact that the second-generation Carl Wilson animals received the stock corn diet fifteen to eighteen days earlier than their parents. But also, it may be reasoned that since these second generation animals had the fluoride supplements fifteen to eighteen days before the first-generation animals, a greater reduction should have occurred in the second-generation animals because they received quantitatively more fluorine, and at an age which apparently produces a greater reduction. There is apparently a balance, then, between starting the animals on a cariogenic diet at an early age with fluorides available and keeping them on a soft noncariogenic

*Composition of diet: granulated sugar, 70 per cent, vitamin test casein, 20 per cent, Wesson oil, 5 per cent, vitamin mixture, 1 per cent, inorganic salt mixture, 4.0 per cent, in addition to percomorph oil each day.

diet until weaning, at which time fluorides are furnished. It should also be pointed out that those animals on the cariogenic diet at an early age have teeth that are undoubtedly less resistant to decay; in fact, McClure[15] indicated this possibility in the statement that "young and newly erupted teeth may be particularly susceptible to decay."

TABLE II. COMPARISON OF THE CARIES-REDUCING EFFECT OF VARIOUS COMPOUNDS IN FIRST- AND SECOND-GENERATION CARL WILSON STRAIN ALBINO RATS

| SUPPLEMENT | NUMBER OF ANIMALS | | NUMBER OF LESIONS | | PER CENT OF REDUCTION | |
|---|---|---|---|---|---|---|
| | FIRST GENERATION | SECOND GENERATION | FIRST GENERATION | SECOND GENERATION | FIRST GENERATION | SECOND GENERATION |
| | | | *Females* | | | |
| $SnCl_2$ (F, 2 ppm) | 16 | 24 | 6.1 ± 0.71 | 7.8 ± 1.06† | 12.8 | 14.2 |
| NaF (F, 10 ppm) | 28 | 20 | 6.0 ± 0.59 | 8.0 ± 0.90 | 14.4 | 12.1 |
| $SnF_2$* (F, 10 ppm) | 29 | 68 | 3.9 ± 0.57 | 6.2 ± 0.63 | 44.3 | 32.0 |
| $H_2O$ (F, 0.15 ppm) | 20 | 36 | 7.0 ± 0.46 | 9.1 ± 1.00 | 0.0 | 0.0 |
| | | | *Males* | | | |
| $SnCl_2$ (F, 2 ppm) | 15 | 30 | 5.3 ± 0.60 | 8.2 ± 0.50 | 32.0 | 3.5 |
| NaF (F, 10 ppm) | 28 | 19 | 5.6 ± 0.52 | 8.1 ± 0.99 | 28.2 | 4.1 |
| $SnF_2$* (F, 10 ppm) | 20 | 48 | 4.2 ± 0.16 | 5.8 ± 0.49 | 46.2 | 31.9 |
| $H_2O$ (F, 0.15 ppm) | 24 | 31 | 7.8 ± 0.51 | 8.5 ± 1.09 | 0.0 | 0.0 |

*A. D. McKay.
†Standard deviation.

TABLE III. COMPARISON OF CARIES INCIDENCE IN FIRST- AND SECOND-GENERATION ALBINO RATS

| STRAIN | GENERATION | FEMALES | | MALES | |
|---|---|---|---|---|---|
| | | NUMBER OF ANIMALS | NUMBER OF LESIONS | NUMBER OF ANIMALS | NUMBER OF LESIONS |
| $MC_1$ | First | 21 | 9.5 | 23 | 8.9 |
| $MC_{2a}$ | Second | 33 | 8.9 | 39 | 8.5 |
| $CW_1$ | First | 20 | 7.0 | 24 | 7.8 |
| $CW_2$ | Second | 36 | 9.1 | 31 | 8.5 |
| $MC_{2b}$ | Second | 40 | 7.0 | 44 | 6.9 |

$MC_{1a}$ are second-generation McCollum strain albino rats raised on stock corn diet from birth; $MC_{2b}$ are second-generation McCollum strain rats placed on a low caries-producing diet at 15 days and kept on this diet for three weeks before being placed on the stock corn diet; these animals received no added supplements.

In an attempt to investigate this further, a group of animals raised in this laboratory (McCollum strain) was divided into two groups. One group ($MC_{2b}$) was placed on a low-cariogenic diet at 15 days of age (which is approximately the time the animals begin to eat solid food), and remained on this diet until 30 days old, after which time they were returned to the cariogenic diet until 170 days old. A second group received the cariogenic diet from birth ($MC_{2a}$) and was used for comparative purposes (see Fig. 1).

Table III demonstrates a comparison of first- and second-generation McCollum and Carl Wilson animals in addition to the McCollum animals that were on the low-cariogenic diet for three weeks ($MC_{2b}$) and their controls, which

were on the cariogenic diet from birth ($MC_{2b}$). It is evident that it is possible to produce a decrease in the caries incidence of the McCollum animals, which had previously shown a higher incidence of decay, by simply providing them with a low cariogenic diet for the first thirty days. For example, the first-generation McCollum female animals ($MC_1$) have 9.5 cavities, and there are 8.9 cavities in the males. It is observed that the second-generation McCollum animals ($MC_{2a}$) which were raised under similar conditions have an incidence of decay of 8.9 cavities in the females and 8.5 in the males when raised on the cariogenic diet from birth. Although these latter values are somewhat lower than the former, they are within the same range, and the difference is probably fortuitous. But when the second generation McCollum animals receive the low cariogenic diet for three weeks prior to being placed on the stock corn diet, ($MC_{2b}$) the incidence of decay is reduced considerably. The first generation Carl Wilson animals had 7.0 cavities in the females and 7.8 in the males, while the second generation had 9.0 cavities in the females and 8.5 in the males. The only difference in the early care of these animals is that the second-generation Carl Wilson animals had eaten the stock corn diet fifteen to eighteen days sooner than the first-generation parents. The second-generation Carl Wilson animals present a decay pattern quite different from the first generation, for in these animals the incidence of decay was very reminiscent of first-generation McCollum animals. If one considers the McCollum animals ($MC_{2b}$) that were treated similarly to the Carl Wilson animals ($CW_1$) before 30 days of age, that is, kept on a soft noncaries-producing diet before being placed on the cariogenic diet, there is noticed a similar reduction in decay in this strain (Table III). The females have 7 cavities, while the males have 6.9. Therefore, this difference in caries incidence of the two strains of control rats does not indicate a strain difference, in our opinion, but is due to biologic variations present in all animals, as well as to the different types of diets which the animals received before weaning.

It has been reported that stannous fluoride reduces the incidence of decay in rats to a greater extent than sodium fluoride at similar stoechiometric equivalents of fluorine ion,[1-3] but in no instance has the effect of sodium fluoride in the males been so low as observed in this experiment. In the first-generation Carl Wilson strain there was a 14 per cent reduction for the females and a 28 per cent reduction in the males, but this report indicates that in the second-generation animals a 12 and 4 per cent reduction was noted in the sodium fluoride females and males, respectively. In the first-generation tin-fluoride animals there was a 44 and 46 per cent reduction in the females and males, respectively, while in the second generation there was approximately a 32 per cent reduction in both sexes. Perhaps this lowered reduction in the latter case may be partially explained by the fact that the second-generation animals received a tin-fluoride preparation which has since been shown to be less effective in reducing caries incidence in rats.[3] Certainly, no similar explanation can account for the difference observed in the sodium-fluoride animals.

It is evident, then, that some consideration should be given not only to the reagent used to inhibit the incidence of decay in rats, but also to the physi-

cal factors of the diet, and the time at which animals are placed on the experimental diet, as well as to the ration they receive before weaning. Previous work[18, 19] has indicated that there is more resistance to decay in an older than in a younger animal, and our data corroborate this finding. Clinically, we are aware that the rate of decay of the young patient appears higher than that of an adult. In this connection, also, there is evidence of a decrease in caries susceptibility with age in human teeth.[20] This work further strengthens this observation, for apparently the first days of eruption of the rats' molars are very important, and if a caries-producing diet is available at an early age (before 30 days) a higher incidence of decay is produced than if the animal is placed on the caries-inducing diet at a later age.

The type of lesions commonly found in the animals which were on the soft-low cariogenic diet for three weeks is of interest. In Sognnaes' report of work with high-sucrose diet fed to rats from birth through tooth development, he stated that the carious lesions he described were more nearly comparable to human caries than any experimental lesions seen or reported in previous publications. He described a typical smooth-surface carious area, as seen occurring in the human subject. Although no such lesions were observed in our animals on the soft-low cariogenic diet, a very different lesion from the ordinary stock corn high-cariogenic type of lesion was observed. These former lesions appear to follow the whole occlusal fissure in destruction of enamel, in contrast to those animals on the stock diet from birth, which presented a more extensive lesion both in size and depth, but appeared to arise from one foci. This is not to be taken to mean that the lesion was necessarily smaller in the former group, but it gave the examiner the impression that the carious process originated over a larger area than did the cavity resulting directly from the stock corn diet. In view of the recent work of Van Huysen,[21] this could be interpreted as a corroboration of his finding, that is, that in a stock corn diet some particles pack down into the fissures, putrify and destroy the apex of the fissures, undermine the enamel, and "fracture" the overlying structures, producing the large area so frequently observed. But in the animals which were on the soft-low cariogenic diet early in life, the situation was frequently quite different. It appeared that instead of the carious process originating at the apex of a fissure, it began by first spreading throughout the entire length of the fissure, and then undoubtedly corn packed into this weakened larger area and developed a cavity quite different in appearance. This later lesion appeared to follow the fissure more generally than it appeared first to undermine the cusp by decay in one area.

## SUMMARY

1. Newly erupted teeth are particularly susceptible to a cariogenic diet. This is indicated by the following: (a) There was a higher incidence of caries in rats on a coarsely ground corn diet as compared with controls given a finely ground low-cariogenic diet until the thirtieth day of age and then fed the coarsely ground corn diet. (b) Apparent strain differences in rats were shown

instead to be due to differences in the diet during the first five weeks. When a low-cariogenic diet was fed during the first four weeks, rats had a rather low incidence of dental caries even when a coarsely ground corn diet was fed for 140 days.

2. Stannous fluoride afforded more protection to rats' caries in the second generation than did either sodium fluoride or stannous chloride, but the degree of protection was considerably reduced over the protection observed in parents (first generation) receiving similar fluoride supplements.

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# EXPERIMENTAL CARCINOGENESIS IN THE CHEEK POUCH OF THE SYRIAN HAMSTER

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SINCE the discovery by Yamagiwa in 1915 that skin cancer could be produced at will by tarring the ear of the rabbit, and since the isolation from tar of the carcinogenic hydrocarbon, benzpyrene, by Cook and Kennaway in 1932, some 1,300 different compounds have been studied for their cancerogenic properties.[6] The literature on experimental carcinogenesis has reached voluminous proportions. However, most of these reports deal with the chemical induction of cancer in regions of the body other than the oral cavity. When it is considered that 8 to 10 per cent of all human cancer occurs in the mouth, and that 4 per cent of cancer deaths in the United States result from intra-oral tumors,[9] it is surprising that so little work of an experimental nature has been done in this seemingly important field.

Of the few reports that do appear, results for the most part have been negative or inconclusive. Levy[11] painted methylcholanthrene on the labial mucosa of mice and hamsters up to sixteen weeks, noting no gross or histologic change in the treated tissues. He also implanted pellets of the same compound at the apex of the lower incisors with a resultant disorderly growth of enamel, dentin, and cementum. In 1950 Levy and Ring[13] succeeded in producing malignant tumors of connective tissue, sarcomas, by implanting crystalline 9,10-dimethyl-1,2-benzanthracene subgingivally in hamsters. Eighty per cent of their animals exhibited tumors after four to five months. Levy, Gorlin, and Gottsegen[12] studied the effect of a single topical application of 9,10-dimethyl-1,2-benzanthracene on the skin and mucous membrane of the lips of mice. The study was terminated after thirty-five days with some premalignant changes noted histologically in the mucous membrane. Wantland[17] sprayed and painted 20-methylcholanthrene, 1,2,5,6-dibenzanthracene, and 2-acetylaminofluorine into the hamster pouch for six weeks with no changes noted other than hyperplasia.

Since 90 to 95 per cent of oral cancer is epithelial in origin,[9, 15] efforts in this study were directed toward attempting to produce this type of tumor. The Syrian hamster *(Cricetus auratus)* was chosen as the most suitable experimental animal for several reasons. It possesses two cheek pouches which communicate with the mouth on either side and which are lined by stratified squamous epithelium similar to that lining the oral cavity proper. The pouch can be readily inverted or pulled out for easy access and gross observation. When in

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this position, it can be studied microscopically by transillumination. Several investigators[5, 10, 14] have utilized this in vivo preparation for studying circulation and transplanted animal and human tumors. It was felt that if squamous cell carcinoma could be experimentally produced in this structure, the hamster would be a very useful tool for more detailed observations of experimental mouth cancer.

The primary purpose of this study, therefore, was to determine the susceptibility of the pouch epithelium to the action of chemical carcinogenic compounds, and to determine the carcinogen of choice for subsequent investigations of initiation, development, and metabolism of induced oral carcinoma.

## EXPERIMENTAL PROCEDURE

A total of thirty-six hamsters, approximately 3 months of age, was obtained from the stock colony maintained at the University of Rochester. A diet of Purina Laboratory Chow supplemented with lettuce was provided with water ad libitum. Unanesthetized animals were immobilized in the device developed by Johansen,[8] the lip of the right side was retracted, and the epithelial surface of the pouch was painted with the solution of hydrocarbon using a No. 4 camel's hair brush. Each pouch was painted three times per week for sixteen weeks with an additional nine-week period of observation. Another group of animals was painted with acetone and benzene to determine the effect of the solvent, and with physiologic saline solution to observe any traumatic effects of brushing. Weights were recorded weekly.

Table I shows the compounds used and the grouping of the animals. They were divided into two series on the basis of the solvent used, and each series was further subdivided into three groups using three hydrocarbons, 9,10-dimethyl-1,2-benzanthracene, 20-methylcholanthrene, and 3,4-benzpyrene. These compounds were chosen since, according to Berenblum[1] and Iball,[7] they are the three most potent compounds for epithelial carcinogenesis in the order listed. They were dissolved in acetone and benzene to compare the effect of the solvent. A 0.5 per cent solution was used in each group.

TABLE I

| SERIES | GROUP | CARCINOGEN | SOLVENT | NUMBER OF ANIMALS |
|---|---|---|---|---|
| | I | 9,10-Dimethyl-1,2-benzanthracene | Acetone | 3♀, 3♂ |
| A | II | 20-Methylcholanthrene | Acetone | 3♀, 3♂ |
| | III | 3,4-Benzpyrene | Acetone | 3♀, 3♂ |
| | IV | 9,10-Dimethyl-1,2-benzanthracene | Benzene | 3♀, 3♂ |
| B | V | 20-Methylcholanthrene | Benzene | 3♀, 3♂ |
| | VI | 3,4-Benzpyrene | Benzene | 3♀, 3♂ |

Animals were sacrificed with chloroform, and whole pouches were dissected out and fixed in Bouin's fluid. After dehydration and embedding in paraffin, serial sections were cut at 7 microns and stained with hematoxylin and eosin

and Mallory's connective tissue stain. Histologic sections of the cervical lymph nodes, tongue, palate, esophagus, forestomach, stomach, lungs, and liver were also made.

## RESULTS

The first change observed grossly was seen in all experimental and control animals painted with benzene solutions or benzene per se. In the initial two weeks of painting, an acute inflammatory reaction developed with subsequent necrosis and sloughing of the distal end of the pouches. The pouch depth, which normally is 3 to 3.5 cm., was reduced to 1 to 1.5 cm. During this period, six of the original eighteen animals in Series B died, as well as two of the six controls. In the surviving animals, the pouches healed with no further evidence of inflammation. Members of the acetone series, experimental and control, did not exhibit these findings.

The first type of neoplasm noted grossly is illustrated in Fig. 1, which shows an inverted pouch containing a papilloma measuring 1 mm. in diameter. Gross morphology of tumors induced was the same regardless of the hydrocarbon used.

Fig. 1.—An inverted pouch (*P*) showing a small papillomatous growth (*arrow*) 1 mm. in diameter. *L*, Lip; *T*, tongue; *I*, mandibular incisors.

Histologically, four degrees of change were seen: hyperplasia, benign papilloma, squamous cell carcinoma in situ (preinvasive), and squamous cell carcinoma with local invasion and metastasis.* Fig. 2 is a photomicrograph of the normal pouch wall. It consists of four distinct layers: flat stratified squamous epithelium, without rete pegs, two to four cell layers in thickness with some keratinization on the surface; a submucosa of dense fibrous connective tissue containing no glands or other accessory structures; a layer of longitudinal striated muscle fibers; and an outer layer of loose areolar connective tissue. Fig. 3 is a section of pouch wall with epithelial hyperplasia, hyperkeratosis, parakeratosis, acanthosis, and some downward proliferation of the epithelium into the submucosa. However, the epithelium is still well stratified, and although

*Diagnoses confirmed by Dr. C. K. Jones, Department of Pathology, University of Rochester, School of Medicine and Dentistry.

there appear to be independent nests of epithelial cells at the bottom center of the photograph, adjacent serial sections revealed these to be connected to the large mass of cells. There was no evidence of cell or nuclear atypicality. Fig. 4

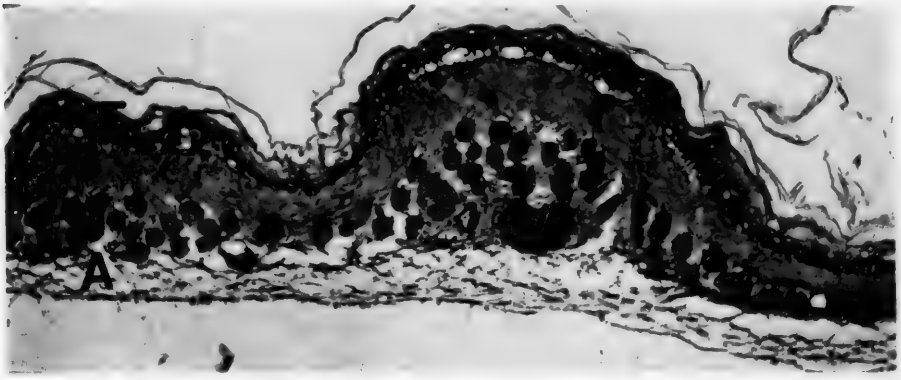


Fig. 2.—Cross section of normal pouch wall. *E*, Epithelium; *S*, submucosal connective tissue; *M*, striated muscle fibers; *A*, loose areolar connective tissue; *V*, blood vessel. (Hematoxylin and eosin. Magnification ×100.)

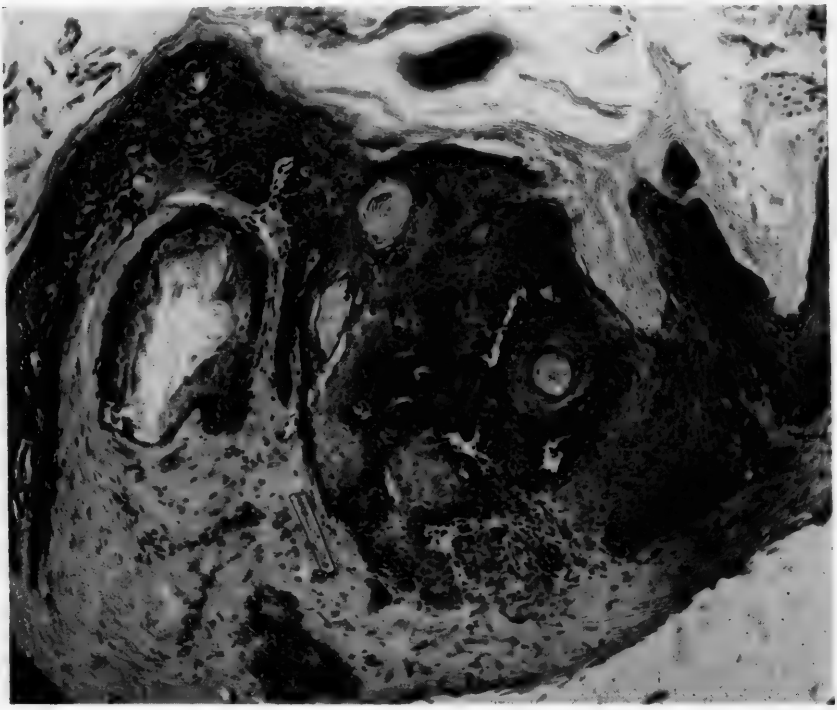

Fig. 3.—Hyperplastic epithelium with keratotic crypts (*K*). No evidence of cell or nuclear atypicality. (Hematoxylin and eosin. Magnification ×100.)

is a papillomatous squamous cell carcinoma in situ. There is a sharp line of demarcation between the benign hyperplastic epithelium on the left and the epithelium on the right, which shows loss of stratification and nuclear atypicality with hyperchromatism. There is extension of the epithelium into the submucosa with numerous abnormal mitotic figures present. Since no evidence of actual invasion could be detected, this type of tumor was designated preinvasive carcinoma.

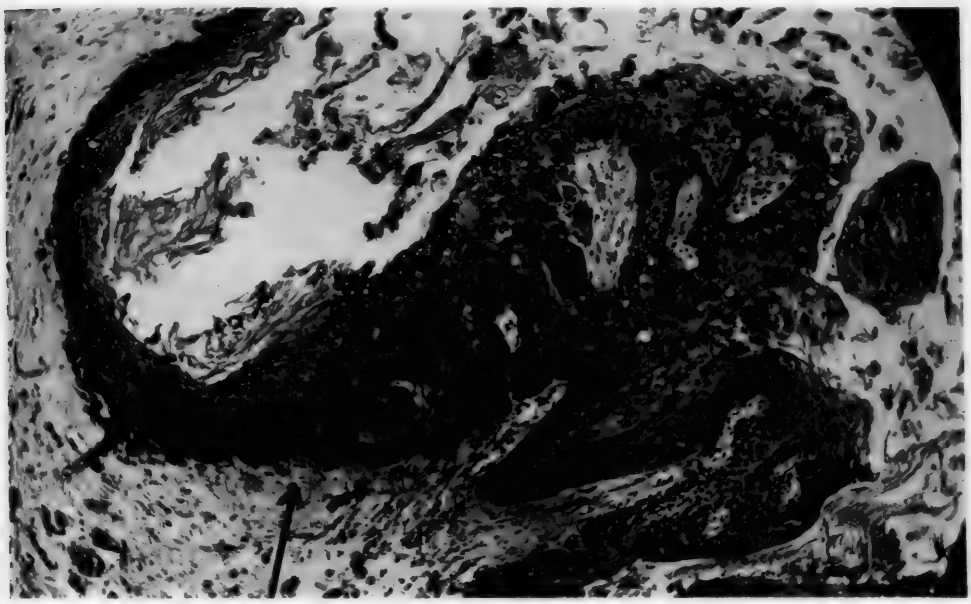

Fig. 4.—Papillomatous squamous cell carcinoma in situ. Note sharp line of demarcation (arrow) between benign and malignant cells. *A*, Artifact. (Hematoxylin and eosin. Magnification ×100.)

Fig. 5 reveals a frank squamous cell carcinoma with nests and sheets of atypical epithelial cells invading the submucosal connective tissue. Fig. 6 is a different field in the same tumor showing nests and cords of wildly growing, abnormal squamous cells scattered throughout the submucosa. A higher magnification of this tumor is shown in Fig. 7. Here can be seen cell and nuclear pleomorphism, nuclei which are hyperchromatic, prominent nucleoli, and some abnormal mitotic figures. As an illustration of one of the most important requirements of malignancy, Fig. 8 demonstrates a cervical lymph node invaded with metastatic squamous cell carcinoma, the primary lesion of which is pictured in Figs. 5, 6, and 7. Normal lymphoid tissue is seen bordering masses of malignant squamous cells revealing marked keratinization. This metastasis is similar to cervical lymph node invasion sometimes seen in human squamous cell carcinoma of the oral cavity.

Gross and histologic examination of other tissues listed previously revealed, in some instances, the presence of neoplasia varying from premalignant hyper-

plasia to squamous cell carcinoma in situ in the tongue, buccal and palatal mucosa, esophagus, and forestomach, all of which are lined with stratified squamous epithelium. This was no doubt due to escape of small amounts of hydrocarbon solution from the pouch into the oral cavity, and thence into the upper portion of the gastrointestinal tract. Pouches of control animals showed no changes, with the exception of the survivors painted with benzene where a slight epithelial hyperplasia was present.

Fig. 5.—Squamous cell carcinoma with invading nests and sheets of cells. (Hematoxylin and eosin. Magnification ×60.)

Table II summarizes the results. It is obvious that 9,10-dimethyl-1,2-benzanthracene in acetone is the most potent carcinogen in the hamster pouch, as all survivors showed squamous cell carcinoma with metastasis, the first evidence of neoplasia being noted at seven weeks. The next in order of potency and time was the benzene solution of the same compound used in Group IV. However, benzene was not a satisfactory solvent because of the high mortality. This is in agreement

Fig. 6.—Local invasion of the tumor in Fig. 5. Note cords of cells invading into connective tissue. (Hematoxylin and eosin. Magnification ×100.)

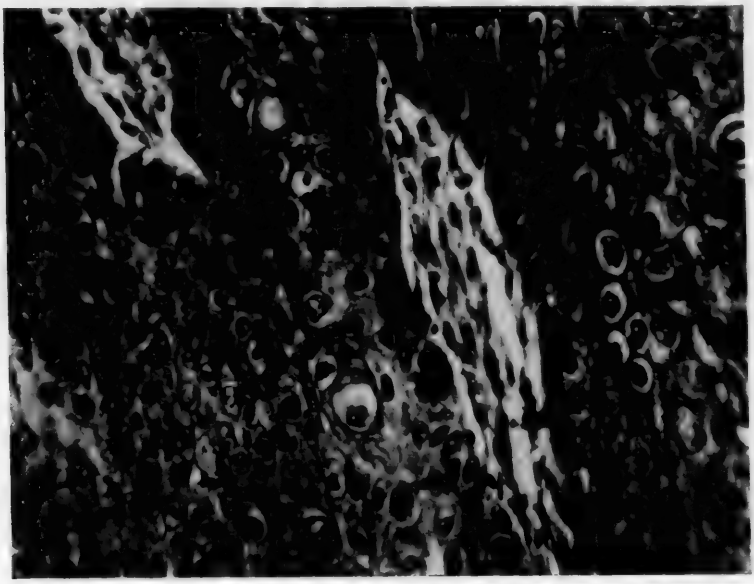

Fig. 7.—High-power view of tumor cells in Fig. 5. (Hematoxylin and eosin. Original magnification ×430, reduced ⅙.)

with the report of Bradbury, Bachman, and Lewisohn,[2] who tested the effect of different solvents and carcinogens on the skin of mice, finding greater toxicity with benzene than with acetone. In Series A, the next in order of potency was 3,4-benzpyrene, where again all survivors had tumors with an average latent period of sixteen weeks. However, one animal had a benign papilloma, two showed squamous cell carcinoma in situ, and only two had carcinoma with metastasis. Methylcholanthrene was last in potency, requiring twenty-five weeks. Four of the five survivors possessed tumors, two of these being benign papilloma, and two preinvasive carcinoma. One animal showed no change. In the twelve animals painted with benzene solutions of 20-methylcholanthrene and 3,4-benzpyrene, only one tumor developed and it was benign in character. According to Bradbury, Bachman, and Lewisohn[2] and Stowell and Cramer,[16] more rapid results are achieved with acetone as a solvent, as it is freely miscible with water, therefore carrying the carcinogen into the cell with greater ease.

Fig. 8.—Cervical lymph node showing metastatic carcinoma from tumor shown in Figs. 5 to 7. *T*, Invading tumor cells; *L*, lymphoid tissue. (Hematoxylin and eosin. Magnification ×100.)

## DISCUSSION

From the data presented in Table II, it can be seen that the hamster pouch is susceptible to the action of the three carcinogenic hydrocarbons employed, depending on the nature of the solvent. As this was a pilot study to determine the susceptibility of the pouch mucosa, only a small number of animals was used in each group, but it can be pointed out that since the completion of this study,

an additional twelve animals have been painted with an acetone solution of 9,10-dimethyl-1,2-benzanthracene to confirm the results obtained in Group I. Of the ten survivors, squamous cell carcinoma with metastasis was induced in all after an average latent period of six to seven weeks. It is felt that similar results could be attained with the other compounds if longer experimental periods were employed. In a description by Cramer and Stowell[3, 4] of the macroscopic and microscopic changes leading to skin cancer induced by methylcholanthrene, the train of events appears to be hyperplasia to papilloma to carcinoma. In this investigation, where a uniform concentration (0.5 per cent solution) was used in all groups, more prolonged application of benzpyrene and methylcholanthrene would probably have resulted in a greater number of tumors with a higher degree of malignancy, since the various steps toward carcinoma were noted with these particular compounds. This and the use of still other carcinogens in the pouch warrant further study.

TABLE II

RESULTS OF CARCINOGENIC TREATMENT ON SURVIVORS*

| | ACETONE SERIES | | | | | BENZENE SERIES | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NO. OF SURVIVORS | NO. WITH BENIGN TUMORS | NO. WITH CARCINOMA | NO. WITH METASTASIS | TIME OF ONSET (WEEKS) | NO. OF SURVIVORS | NO. WITH BENIGN TUMORS | NO. WITH CARCINOMA | NO. WITH METASTASIS | TIME OF ONSET (WEEKS) |
| 9-Dimethyl-2-benzanthracene | 5 | 0 | 5 | 5 | 7 | 4 | 0 | 3 | 3 | 8 |
| Methylcholanthrene | 5 | 2 | 2 | 0 | 25 | 3 | 1 | 0 | 0 | 25 |
| 3-Benzpyrene | 5 | 1 | 2 | 2 | 16 | 4 | 0 | 0 | 0 | — |

*Original number in each group, 6.

With the knowledge that the hamster pouch can be utilized successfully for the experimental induction of squamous cell carcinoma, more detailed investigations are now in progress to gain some insight into the in vivo gross and microscopic changes resulting from the topical application of these compounds.

## SUMMARY

A technic has been described for the production of malignant epithelial tumors in the cheek pouch of the Syrian hamster using three of the polycyclic hydrocarbons. Tumors ranging from benign papilloma to squamous cell carcinoma with lymph node metastasis were produced. An acetone solution of 9,10-dimethyl-1,2-benzanthracene proves to be the best combination. 3,4-Benzpyrene and 20-methylcholanthrene in acetone also gave positive results, but to a lesser degree. The effects of solvents, duration of painting, and the possible use of the hamster in more detailed studies of experimental oral cancer are briefly discussed.

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# THE INFLUENCE OF INJURY TO THE PERIODONTAL MEMBRANE ON THE SPREAD OF GINGIVAL INFLAMMATION

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## INTRODUCTION

IN A previous report, the early histologic changes in the supporting tissues of rat molars during experimental tooth movement have been described (Macapanpan, 1952). Tooth movement had been induced by the insertion of a piece of rubber dam between the upper first and second molars. This rubber dam acted not only as a separator but also as a foreign body. Its insertion led to severe papillary gingivitis that later spread into the adjacent tissues. The result of this experimental procedure thus was twofold: by local injury, it produced a gingivitis, and by a movement of the tooth exceeding physiologic limits, it produced a simultaneous damage to the periodontal membrane. It was, therefore, thought worth while to analyze the spread of a gingival inflammation in instances of coexisting trauma to the periodontal membrane.

## MATERIAL AND METHOD

*Material.*—This study is based on the histologic examination of serial sections of the teeth and supporting tissues of thirty-five male rats of the Sprague-Dawley strain, 65 to 70 days of age. The animals were fed Purina Chow ad libitum.

*Method.*—A piece of rubber dam, 1 mm. in width, was inserted between the upper right first and second molars. The left side of the upper jaw was used as control. Four animals each were sacrificed after one, three, six, twelve, twenty-four, thirty-six, forty-eight, and sixty hours, and three animals after seventy-two hours. After fixation in 5 per cent neutral formalin and decalcification in 25 per cent formic acid, the specimens were embedded in celloidin, serially sectioned, and stained with hematoxylin and eosin. For each experimental period, two specimens were sectioned horizontally, and two were sectioned mesiodistally.

## FINDINGS

The pressure of the rubber dam between the first and second molars moved the first molar mesially, the second and third molars distally (Fig. 1). The movement of the second and third molars was less pronounced than that of the first molar. All these teeth were shifted bodily so that the entire periodontal membrane of all roots was widened on the side of tension and narrowed on the

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side of pressure. These changes in the thickness of the periodontal membrane were especially marked around the first molar, so much so that necrosis in some areas of the periodontal membrane on the side of pressure could be observed.

In the region between the first and second molars, the site of the moving force, the periodontal membranes on either side of the interdental septum were areas of tension and therefore widened, although more at the first molar than at the second (Figs. 1 through 6).

*One to Twelve-hour Experimental Period.*—The gingival papillae between the first and second molars were compressed and considerably flattened by the rubber dam, the distance between the epithelium and the crest of the interdental septum thus being reduced. The epithelial attachments did not proliferate, and remained on the enamel, with their apical ends at the cemento-enamel junction. The gingival epithelium also remained intact. A few leukocytes could be seen immediately beneath it. In rare instances, leukocytes could be observed in the deeper layers of the connective tissue close to the alveolar crest and in the immediate vicinity of blood vessels.

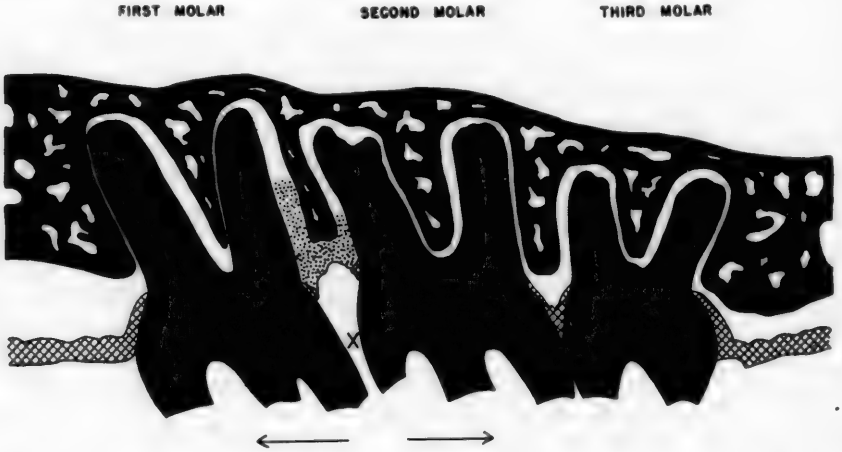



Fig. 1.—Semidiagram showing an anteroposterior section through upper molars of white rat. Arrows indicate direction of movement induced by rubber dam inserted at area *X*. Note ulceration of the papilla between the first and second molars. The first molar moved mesially, the second and third molars distally, as indicated by the compression of the periodontal membranes facing mesial surfaces of roots of the first molar and distal surfaces of roots of second and third molars. Infiltration has extended deeper into more widened periodontal membrane on mesial side of interdental septum between first and second molars than in less widened periodontal membrane on distal side of the septum.

*Twenty-four to Seventy-two Hour Experimental Period.*—Compression of the gingival papillae by the rubber dam became increasingly apparent during this period (Figs. 5 and 6). In most instances, the surface epithelium had disappeared. However, the epithelial attachment on the first molar was in many cases still in contact with the enamel, with its apex at the cemento-enamel junction. In other cases, it also was destroyed, and even the gingival fibers were stripped from the cementum. The epithelium on the second molar remained firmly attached in all cases and had its apex at the cemento-enamel junction.

During this period, the crest of the septum between the first and second molars, normally the site of appositional growth (Figs. 3 and 4), frequently showed osteoclastic resorption that gradually increased in intensity (Figs. 5 and 6). The widened periodontal membranes on the tension sides of the molars showed a large number of young connective tissue cells, among them many mitotic figures. The fiber bundles of the periodontal membrane were markedly stretched, especially near the alveolar crest. The blood vessels were wide and engorged. In some animals, capillary damage and small hemorrhages could be observed.

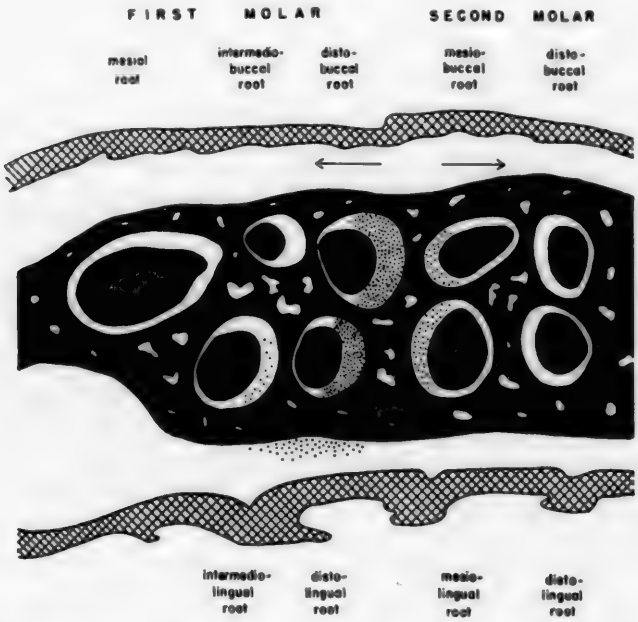
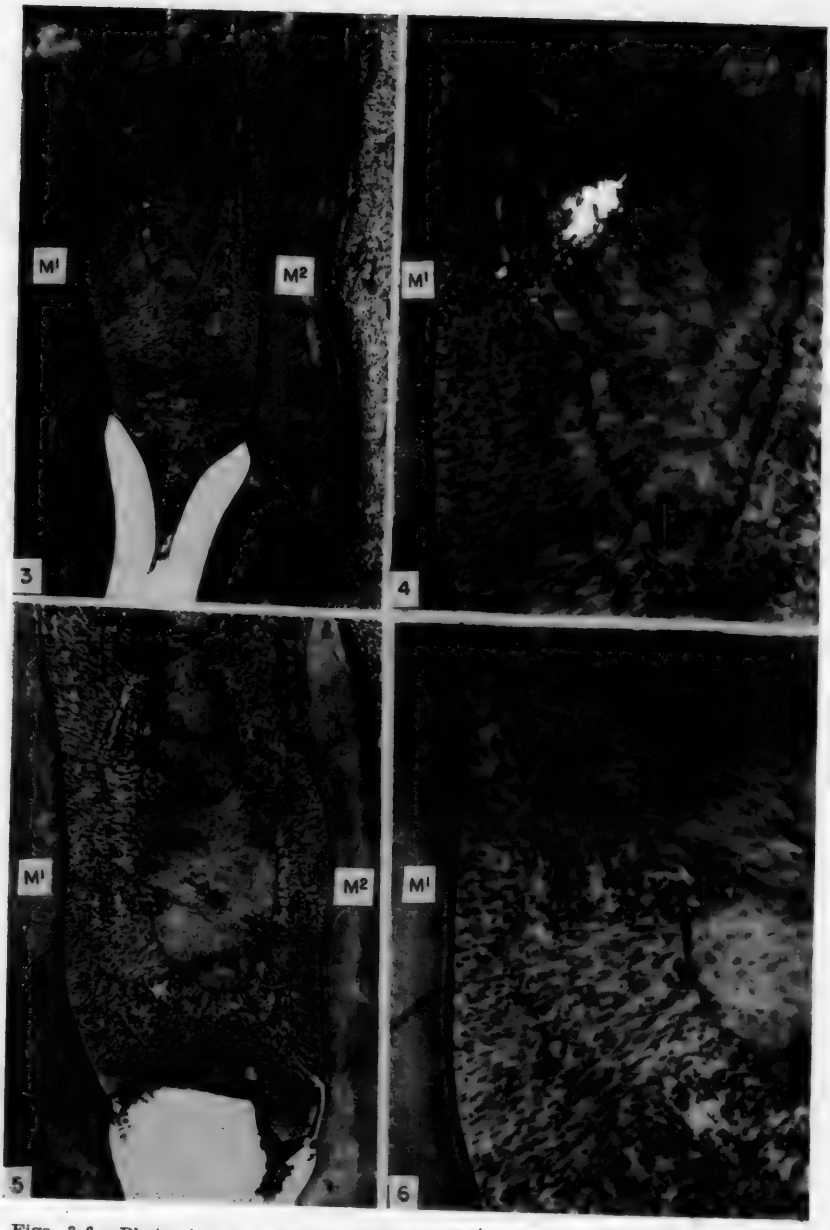



Fig. 2.—Semidiagram of cross section through first and second molars of white rat. Arrows indicate movement of teeth. Note compression of periodontal membranes on mesial surfaces of roots of first molar and on distal surfaces of roots of second and third molars, and widening of periodontal membranes facing distal surfaces of roots of first molar and mesial surfaces of roots of second and third molars. Note more severe inflammatory infiltration into more widened periodontal membrane of the distal roots of first molar than into narrower periodontal membrane of mesial roots of the second molar, and infiltration into lingual attached gingiva and into periodontal membrane of intermediolingual root. Other periodontal membranes are free of infiltration.

The slight papillary gingivitis observed in the first twelve hours had increased in severity. In some cases, the gingival connective tissue showed an accumulation of many inflammatory cells, mainly of heterophil leukocytes and some macrophages. After one day, the gingivitis had developed into a periodontitis. Heterophil leukocytes had infiltrated into the periodontal membranes on the distal surface of the distal roots of the first molar, the sites of greatest widening of the periodontal spaces in the interdental area. By the end of the second day, single leukocytes reached the junction of the cervical and middle thirds of the periodontal space, and on the third day, they could be traced to




Figs. 3-6.—Photomicrographs showing the interdental areas between the first ($M^1$) and second ($M^2$) molars. Figs. 3 and 4, control; Figs. 5 and 6, rubber dam has been placed between first and second molar for sixty hours. Figs. 3 and 5, original magnification ×100, reduced 2/5; Figs. 4 and 6, high magnification (original magnification ×180, reduced 2/5) of the cervical portion of periodontal membrane of first molar.

Fig. 3.—The gingival papilla fills the interdental space and the gingival epithelium is intact. The periodontal membrane distally to the first molar is thinner than the periodontal membrane on the mesial side of the second molar. The crest of the septum shows bone apposition.

Fig. 4.—The periodontal membrane is free from inflammatory cells.

Fig. 5.—The gingival papilla is flattened and ulcerated. The gingival connective tissue shows an accumulation of polymorphonuclear leukocytes and macrophages which have infiltrated more deeply into the more widened periodontal membrane of the first molar than into the less widened periodontal membrane of the second molar. Note also the resorption of the alveolar crest.

Fig. 6.—There is gingival inflammation and leukocytic infiltration into the periodontal membrane and osteoclastic resorption of the alveolar crest.

about the middle of the root (Figs. 5 and 6). The papillary gingivitis also spread into the periodontal membrane on the mesial surface of the second molar. However, the inflammation was less severe and the infiltration penetrated much less deeply than in the periodontal membrane of the first molar. For instance, at a time when the cervical half of the periodontal membrane on the mesial side of the septum was involved, the leukocytic infiltration had affected only one-third of the periodontal membrane on the distal surface (Fig. 1).

During this time, the inflammation of the gingival papillae had spread not only into the periodontal membrane, but also to the marginal gingiva on the lingual side of the first molar mesially to the level of the bifurcation between the distal and intermediate roots (Fig. 2). In some instances, infiltration on the lingual side had progressed into the periodontal membrane distal to the intermediolingual root, that is, into an area of tension. On the corresponding buccal side, however, inflammation did not extend from the site of insertion of the rubber dam, but remained confined to the papilla. The periodontal membrane on the distal (tension) side of the intermediobuccal root was free of leukocytic infiltration.

It was difficult to ascertain inflammation in the marrow spaces because of the hemopoietic nature of the marrow.

## DISCUSSION

The findings in this experiment contribute to the answers to three questions:

1. What is the damage to the periodontal membrane caused by trauma?

2. What influence does damage from such a cause have on the progress of a coexistent gingival inflammation?

3. Does traumatic injury to the periodontal membrane, by itself, cause an inflammatory reaction?

*Injury to the Periodontal Membrane Resulting From Trauma.*—Damage to the periodontal membrane by lateral compression has been recognized repeatedly and has been described in detail (Sandstedt, 1904, 1905; Oppenheim, 1911, 1912, 1933, 1934, 1935; Johnson, Appleton, and Rittershofer, 1926; Orban, 1928; Gottlieb and Orban, 1931, 1938; Stuteville, 1938; Reitan, 1951). Interference with blood supply, hyalinization, and compression necrosis of the periodontal membrane and sometimes even of adjacent alveolar bone have been described as consequences of traumatic forces in areas of pressure. Changes secondary to these consequences, such as, undermining resorption of the alveolar bone in such areas, removal of tissue debris by macrophages, and, finally, anatomic and functional regenerative restitution, have been observed by the same authors.

Although, on the side of tension, proliferation of the connective tissue cells, hemorrhages in some areas, and increased bone apposition had been observed by Gottlieb and Orban (1931), severe damage on the side or in areas of tension has been noted only when strong forces have been applied. Following such procedures, vascular damage and hemorrhages have been described (Sandstedt, 1904; Oppenheim, 1911). Not much attention has been paid to the less obvious damage on the tension side which results from the use of less strong forces.

It stands to reason, however, that when movement of a tooth leads to severe compression or even obliteration of the periodontal membrane on one side, it must also cause damage to the bundles of periodontal fibers in the corresponding areas of tension. The principal fibers of the periodontal membrane are arranged in such fashion that they arrest masticatory movements of the teeth before a damaging degree of pressure is caused. They are, therefore, necessarily injured when a tooth is moved beyond the range of physiologic positions. Actually, experimental damage of the periodontal fibers is hardly ever visible under the microscope, but this is, in all probability, due to the structure of the alveolodental ligaments (bundles of principal fibers). It appears almost certain that the single principal fiber does not extend from bone to cementum, but that shorter fibers are "spliced together" to form these ligaments. If this is correct, then injury to such ligaments would result in unraveling or loosening of the single fibers at many points in the "rope," leading to a stretching of the bundles rather than to a tear of the whole strand. Previous investigations, as well as the present one, showed marked stretching of the periodontal fiber bundles.

Trauma thus causes damage to the periodontal membrane not only on the pressure but also on the tension side.

Some authors regard any repair of tissue after an injury as an inflammatory process (Menkin, 1950). This definition of inflammation seems unnecessarily broad. Repair of injured bundles of periodontal fibers occurs by proliferation of fibroblasts and formation of new fibers, with simultaneous apposition of bone. Repair on the pressure side is accomplished by osteoclastic resorption of bone and proliferation and reorganization of connective tissue. These processes have to be classified as regeneration.

*The Spread of Gingival Inflammation.*—The progress of a gingival inflammation in the absence of injury to the periodontal membrane has been the subject of many investigations, which have led to conflicting conclusions. One school of thought holds that inflammation of the gingiva progresses directly into the periodontal membrane either by way of the lymphatics (which were held to pass from the gingivae to the periodontal membrane along the fiber bundles) or through the haversian canals (Talbot), 1899; Merritt, 1931; Black, 1936; Fish, 1939). Noyes (1937) and Coolidge (1931) agreed that there is usually direct invasion along the periodontal fibers. They held that only in exceptional cases does an inflammation invade the bone first and then reach the periodontal space.

The opposing view, according to which direct invasion of the periodontal space is the exception and infiltration of the bone is the usual pathway of a spreading gingivitis, is held by James and Counsell (1927), Thoma and Goldman (1937), Box (1940), and Kronfeld (1949).

Zuckerkandl (1891) demonstrated that the periodontal membrane is supplied by nerves and blood vessels from interdental and interalveolar arteries and is drained into the interdental veins and lymph vessels. These structures form a bundle which is surrounded by loose connective tissue and which lies in a relatively wide interdental canal that opens at the alveolar crest. Weinmann (1941), in re-examining the mode of propagation of inflammation into the

deeper supporting tissues, pointed out that these anatomic findings explain the peculiar route of inflammatory progress in man. The loose connective tissue which surrounds the neurovascular bundle in the interdental canal constitutes the path of least resistance, and a gingivitis will therefore, as a rule, spread along it rather than into the dense connective tissue of the periodontal membrane, which offers more resistance. The periodontal membrane is not invaded primarily but is reached after destruction of some of the alveolar bone, which occurs after invasion of the marrow spaces of the septum. Likewise, marginal gingivitis does not easily spread into the buccal or lingual areas of the periodontal membrane but rather into the attached gingiva and from there along the periosteal surface of the alveolar process. Only occasionally was a direct invasion of the periodontal membrane observed, but no cause for this exceptional behavior could be ascertained at that time.

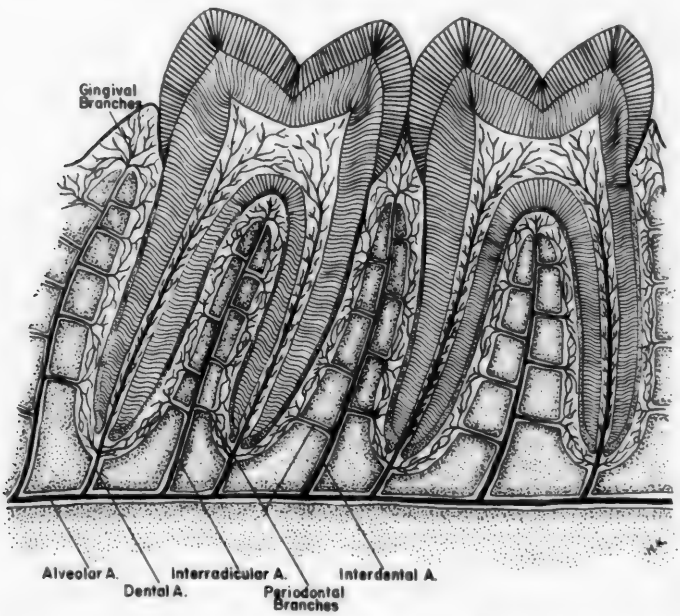



**Fig. 7.—Diagram showing the distribution of the arteries supplying the human periodontal membrane and gingiva. (Courtesy of H. Sicher, from Noyes, F. B., Schour, I., and Noyes, H. J.: Dental Histology and Embryology, Philadelphia, 1938, Lea & Febiger.)**

The distribution and disposition of the blood and lymph vessels and nerves in the molar region of a rat are very similar to those in the human jaws (Fig. 7). An inflammation originating in the gingival papillae should therefore be expected to follow the same pattern of propagation. Instead, leukocytic infiltration of the papillary gingiva observed in the present series of experiments soon spread directly into the periodontal membrane and did not follow a perivascular course into the marrow spaces of the interdental septum. The experi-

mental procedure resulting in direct damage on the tension side of the periodontal membrane may explain these observations. Injury of this kind would eliminate or at least diminish the high resistance of a normal periodontal membrane and thus open the way for a direct involvement of the periodontal space. An analogous mechanism may be operative in man in cases of injury to the periodontal membrane by chronic food impaction, ill-fitting orthodontic bands, and so forth. In such cases, a papillary gingivitis of local origin might, when it increases in severity, produce a periodontitis by spreading directly into the periodontal membrane. Injury to the periodontal membrane of this or a similar type may have been responsible for the exceptional cases of direct invasion of the periodontal membrane observed by Weinmann (1941).

*Absence of Inflammation in Traumatized Periodontal Membrane.*—That traumatism of the periodontal membrane, by itself, can cause an inflammatory reaction was believed and still is maintained by some authors (Stillman and McCall, 1922; Miller, 1950) and is denied by the findings and interpretations of others, especially those of Gottlieb and Orban (1934). The findings in the present investigation suggest that traumatism will be followed by regeneration, and alone will not cause gingivitis or periodontitis, but will merely facilitate and direct the spread of a gingival inflammation already present into the adjacent supporting tissues, although the short duration of the present experiment precludes a full answer to the question.

The mesial movement of the first molar entailed as much widening of the periodontal membrane on the distal surfaces of the intermediate roots as on the distal surfaces of the distal roots. This means that corresponding areas of the periodontal membrane in these roots are damaged to the same degree. Now, the inflammation originating in the interdental papilla spread mesially along the lingual marginal gingiva and reached the bifurcation between the intermediolingual and distolingual roots. At the end of the experimental period, it had progressed from here into the periodontal membrane on the distal side of the intermediolingual root. The corresponding gingiva on the buccal side remained free of inflammatory invasion during the time of observation. The periodontal membrane of the intermediobuccal root, although evidently damaged to the same degree as that of its lingual neighbor, was entirely free of inflammatory cells, and showed purely regenerative processes. Thus, traumatism, by itself, did not cause inflammatory changes.

## SUMMARY

The progress of gingivitis of local origin into the periodontal membrane damaged during tooth movement was studied in serial histologic sections of the molar teeth and supporting tissues of thirty-five male young adult rats of the Sprague-Dawley strain. Tooth movement was induced by the insertion of a piece of rubber dam between the upper right first and second molars. The rubber dam acted also as a foreign body and caused papillary (interdental) gingivitis, characterized by massive leukocytic infiltration that later spread into the deeper supporting tissues, thus producing a periodontitis. The rubber dam was allowed to remain in place from one to seventy-two hours.

The histologic findings were:

1. In the first twelve hours, compression of the gingival papilla and mild inflammation immediately beneath the gingival epithelium were observed. In a few cases, the inflammation extended into the deeper layers of the connective tissue close to the alveolar crest.

2. Between twenty-four and seventy-two hours, the gingivitis increased in severity and progressed into periodontitis. The inflammation spread directly into the periodontal membrane injured by tension on both sides of the interdental septum between the first and second molars. However, the inflammatory cells infiltrated deeper into the more severely damaged periodontal membrane of the first molar. Inflammation also extended mesially into the lingual margin and attached gingiva, reaching the septum between the distolingual and intermediolingual roots of the first molar. From here it spread into the periodontal membrane of the intermediolingual root. On the corresponding buccal side, however, the inflammation remained confined to the papilla, and the periodontal membrane of the intermediobuccal root remained free of inflammatory cells.

## CONCLUSIONS

1. Trauma causes damage to the periodontal membrane on both pressure and tension sides.

2. Damage to the periodontal membrane on the tension side diminishes the resistance of the normal periodontal membrane to direct infiltration from a gingival inflammation leading to periodontitis.

3. The findings in this experiment support the view that traumatism of the periodontal membrane will not, by itself, cause periodontitis.

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# HISTOCHEMICAL EVALUATION OF GINGIVAL HEALING IN EXPERIMENTAL ANIMALS ON ADEQUATE AND VITAMIN C DEFICIENT DIETS

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THE effect of vitamin C deficiency on wound healing in areas other than the oral cavity has been studied using histochemical techniques.[1-3] However, these techniques have not been employed to study gingival healing. Because histochemical staining techniques reveal features of the gingiva which are not detectable by routine staining methods,[4] it was felt that the former methods should be applied to the study of normal gingival healing as well as the manner in which it might be affected by acute vitamin C deficiency.

## PROCEDURE

Gingivectomy was employed as the operative procedure to provide gingival tissue in various stages of healing.

Forty-four guinea pigs, nineteen males and twenty-five females, weighing 224 to 362 grams, were divided into three groups, as follows: Group I, complete diet and gingivectomy, Group II, vitamin C deficient diet,* and Group III, vitamin C deficient diet and gingivectomy. The animals in all groups received the same vitamin C deficient diet. In Group I, the diet was supplemented with daily injections of 10 mg. of ascorbic acid.

After Groups II and III had been maintained on the vitamin C deficient dietary regime for fourteen days, clinical signs of scurvy were evident. The animals were listless, dragged their limbs, and presented alopecia and crusting of the eyelids. Gingival biopsies were obtained from the animals in Group II after eighteen and twenty-eight days on the experimental diet in order to study the changes which resulted from the deficiency.

After fourteen days on the vitamin C deficient diet, a gingivectomy was performed on each of the animals in Group III. At four, seven, ten, fourteen, eighteen, twenty, and twenty-one days after the operation was performed, specimens of healing gingiva were removed. For purpose of comparison, gingivectomies were also performed on the animals in Group I (adequate diet), and specimens of healing gingiva were removed at similar postoperative intervals.

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*The vitamin C free diet was made up as follows: whole milk powder, 1,363.5 grams; ground rolled oats, 831 grams; bran, 831 grams; brewer's yeast, 165 grams; cod liver oil, 17.7 c.c.; NaCl, 41.6 grams; ferric citrate, 13.8 grams. The whole milk was heated in a dry oven at 120° C. for two hours before being combined with the mixture.

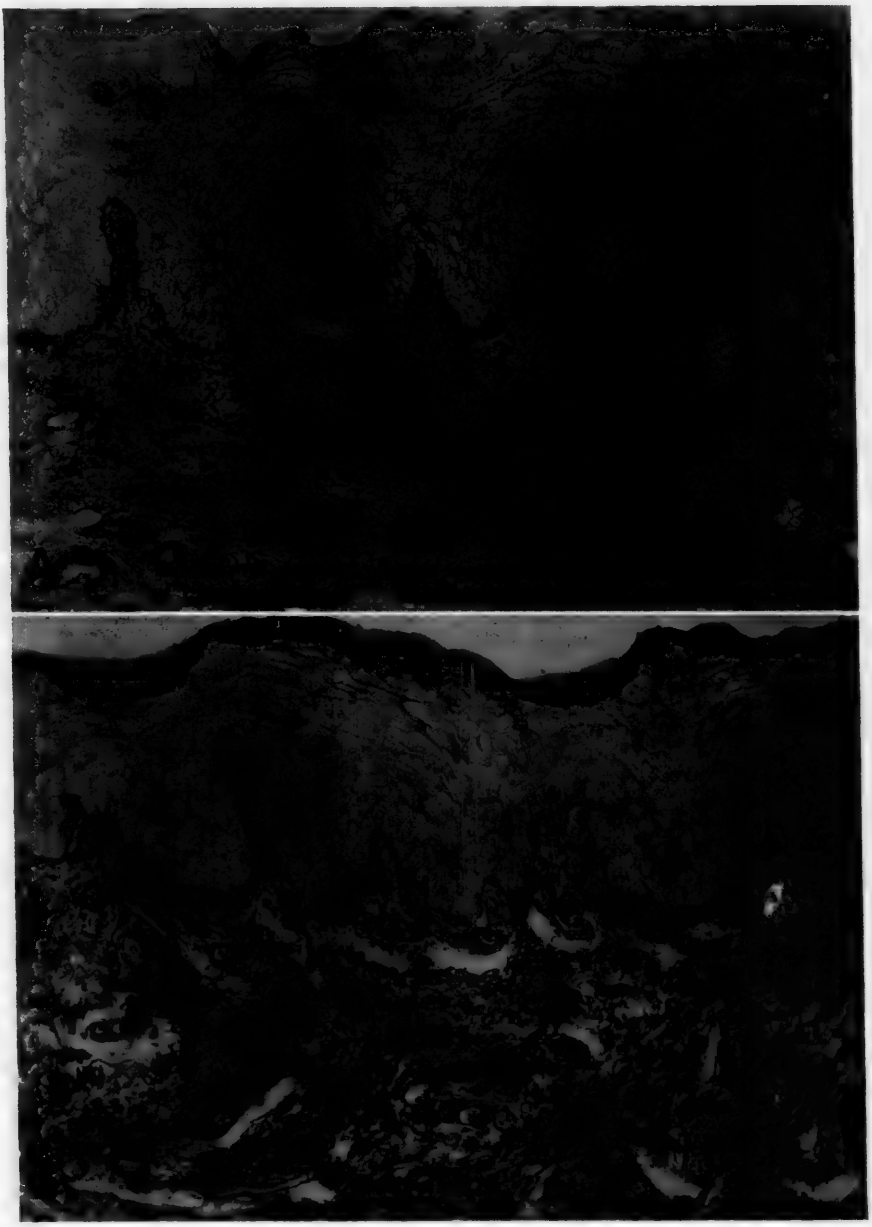

Fig. 1.—*A*, Gingival biopsy of guinea pig on complete diet showing basement membrane, ground substance, and blood vessels in the connective tissue. (Lillie's periodate-leucofuchsin method. Original magnification ×150, reduced ⅛.)

*B*, Gingival biopsy of guinea pig on vitamin C deficient diet for eighteen days showing edema, reduction in ground substance, beading and fragmentation of collagen fibers, and engorgement of blood vessels. (Lillie's periodate-leucofuchsin method. Original magnification ×150, reduced ⅛.)

All tissue was prepared for microscopic study in accordance with the Lillie histochemical technique.[5] The tissue was fixed in either chilled absolute alcohol for forty-eight hours at 5° C., or in a solution of alcoholic Formalin for twenty-four hours. The latter fixative was employed in the preparation of some of the tissues in an effort to obtain better preservation of the glycogen. However, comparable results with glycogen were obtained with both fixatives. Serial sections were stained for ground substance, basement membrane, and glycogen. Controls were conducted for free aldehydes and stainable iodates. For the purpose of orientation, a section of each specimen was stained with hematoxylin and eosin.

At the termination of the postoperative experimental period, the diet of vitamin C deficient-gingivectomy animals (Group III) was supplemented with ascorbic acid, 10 mg. daily for a period of ten days.

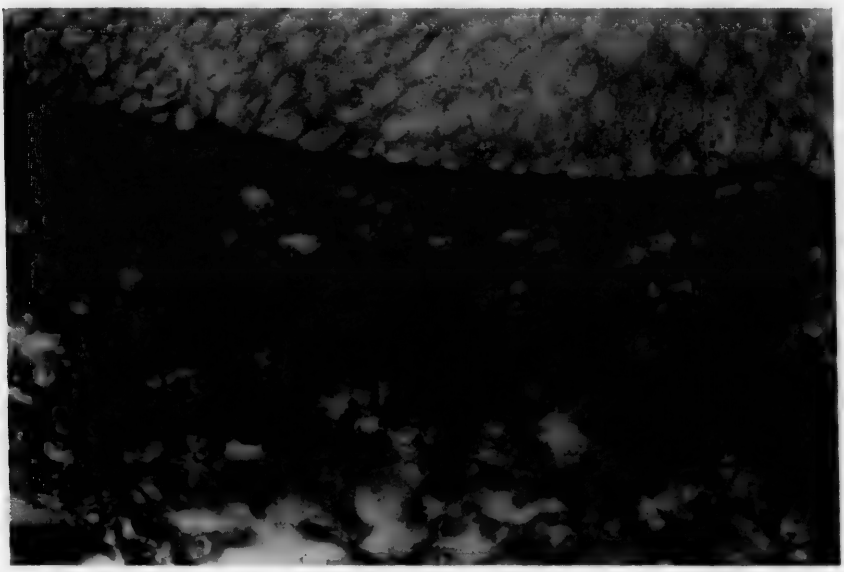

Fig. 2.—Healed gingiva of animal on complete diet, fourteen days after gingivectomy, showing well-defined deeply staining basement membrane and homogenous ground substance in the connective tissue. (Lillie's periodate-leucofuchsin method. Original magnification ×150, reduced ¼.)

With the preceding experimental procedure, it was possible to study the following: normal gingiva, gingiva in vitamin C deficient animals, and various stages of gingival healing in animals on adequate and vitamin C deficient diets.

## FINDINGS

*The Effect of Vitamin C Deficiency on the Gingiva.*—After eighteen days on the vitamin C deficient diet, the gingiva presented a diffusely edematous appearance. Microscopically, comparison of the unoperated gingiva in the control and vitamin C deficient animals revealed no notable difference until

Fig. 3.—Vitamin C deficient animal diet, fourteen days after gingivectomy. Low-power survey of ulcerated incompletely healed area. (Lillie's periodate-leucofuchsin method. Original magnification ×30, reduced ⅛.)

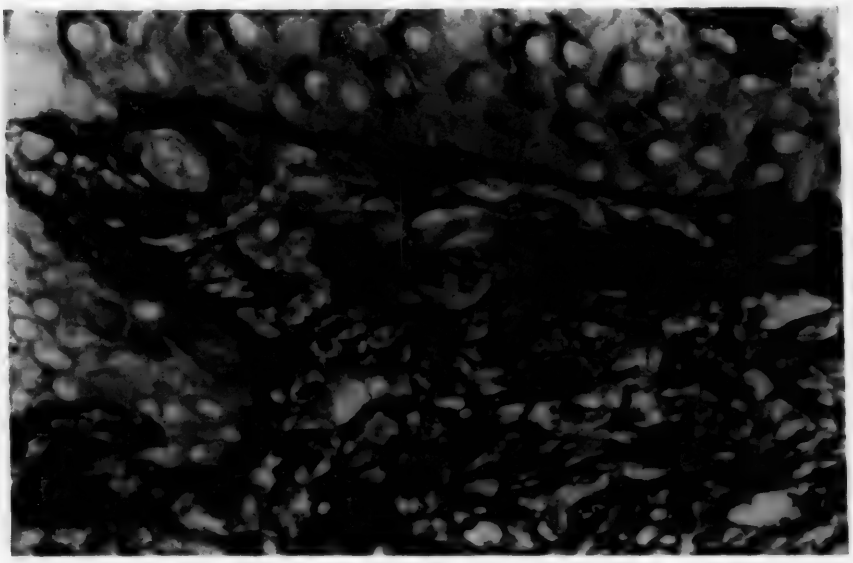

Fig. 4.—High-power study of Fig. 3 (area marked with arrow), showing broad, ill-defined, deeply staining basement membrane and amorphous patches of procollagen with thin strands of collagen. Compare with Fig. 2. (Lillie's periodate leucofuchsin method. Original magnification ×150, reduced ¼.)

the eighteenth day on the vitamin C deficient diet (Fig. 1). At that time, the connective tissue of the gingiva in the vitamin C deficient animals presented edema, and reduction in the ground substance and glycogen. The collagen fibers were more prominent, and appeared beaded and fragmented. The blood vessels were dilated and engorged, and the vessel walls appeared wavy and slightly thickened. There were no changes in the gingival epithelium of the vitamin C deficient animals.

*Gingival Healing in the Control and Vitamin C Deficient Animals.*—Comparison of the postgingivectomy healing in the adequate-diet group and vitamin C deficient group revealed the following: The deficient-diet animals healed comparatively slowly with crust formation rather than surface closure. Whereas the operated area in the adequate-diet group showed complete surface epithelization in ten days, and attained completely normal appearance in eighteen days, the gingivectomy wounds in the deficient animals were still covered by a crust and exudate at the twenty-first postoperative day. Throughout the experimental period the gingiva in the operated area in the vitamin C deficient animals was much more friable than that in the control animals.

Comparison of the microscopic appearance of healing gingiva in the control and vitamin C deficient animals is presented in Table I. In both the control and the vitamin C deficient animals in the early stages of healing, there was microscopic evidence of inflammation with associated degenerative changes and surface ulceration. In the control animals, the inflammation did not persist in any appreciable degree beyond the fourteenth day. In the vitamin C deficient animals, inflammation and ulceration persisted throughout the twenty-one day postoperative period.

The vitamin C deficient gingivectomy animals which were given ascorbic acid at the end of the experiment appeared normal clinically and showed microscopic evidence of normal gingival healing after ten days on the ascorbic acid regime.

## DISCUSSION

This study provided an opportunity to supplement pre-existent knowledge, derived from routine staining methods, regarding the effect of acute vitamin C deficiency on the gingiva. It also affords a basis for analysis of the effects of vitamin C deficiency on gingival healing in terms of histochemically detectable changes.

The effect of acute vitamin C deficiency on the unoperated gingiva was confined to the connective tissue, and consisted of reduction in ground substance and glycogen with beading and fragmentation of the collagen fibers. It is noteworthy that the histochemical methods employed in this study corroborate the previous observation that, whereas degenerative gingival changes are associated with acute vitamin C deficiency, gingival inflammation is not necessarily a concomitant finding.[7]

The retardation in the rate of gingival healing in acute vitamin C deficiency and the apparently increased susceptibility of the wounds to infection observed

TABLE I. COMPARISON OF GINGIVAL HEALING IN ANIMALS ON ADEQUATE AND VITAMIN C DEFICIENT DIETS

| TIME | EPITHELIUM | | CONNECTIVE TISSUE | | BASEMENT MEMBRANE | |
|---|---|---|---|---|---|---|
| | CONTROL ANIMALS | VITAMIN C DEFICIENT ANIMALS | CONTROL ANIMALS | VITAMIN C DEFICIENT ANIMALS | CONTROL ANIMALS | VITAMIN C DEFICIENT ANIMALS |
| 4-10 days | Intercellular substance stained throughout, less prominent in basal layer<br><br>Sparse intracellular distribution of glycogen in granular and prickle cell layers, glycogen concentrated in areas of inflammation; also present in polymorphonuclear leukocytes | Same as control | Ground substance noted on fourth day; very thin, lightly staining strands of collagen fibers visible in ground substance backgrounds; isolated patches of red-staining procollagenous material also appeared on fourth day; scattered fibroblasts with purple-staining glycoprotein granules resistant to treatment with diastase; numerous blood vessels | Absence of ground substance and relatively few blood vessels; the remainder of the features are essentially the same as in the controls | Broad, ill-defined, deeply stained | Broad, ill-defined, deeply stained |
| 14-18 days | Intact epithelial covering with edema of basal and prickle cells | Cellular degeneration and polymorphonuclear leukocytic infiltration | Compared with 4-10 days, ground substance denser and collagen coarser; edema present in papillary layer (Fig. 2) | Same as 4-10 days (Figs. 3-4) | Well-defined, deeply stained (Fig. 2) | Same as 4-10 days (Figs. 3-4) |
| 20-21 days | Intact epithelial covering | Same as 14-18 days | Mature connective tissue with well-formed ground substance, collagen fibers, and fibrocytes | Same as 4-10 days | Thin, well-defined, and less deeply stained | Same as 4-10 days |

in this experiment were in accord with previous studies of the effect of vitamin C deficiency on the healing of other tissues.[2, 8-12] However, several hitherto undescribed aspects of normal gingival healing and the manner in which it is affected by acute vitamin C deficiency were revealed by the histochemical technique employed in this study.

In the healing gingiva of the control and vitamin C deficient animals, the normally sparse glycogen content of the epithelium was increased in areas of hyperplasia associated with inflammation. This increase in glycogen associated with epithelial activity was apparently unaffected by the vitamin C deficiency.

In the healing gingiva of the control animals, ground substance made its first appearance on the fourth day as a thin amorphous film overlying thin collagen fibers. During the later stages of healing, the ground substance was denser and the collagen fibers were coarser, but less prominent against the ground substance background. In the scorbutic animals, there was no formation of ground substance throughout the healing period. Instead, there were gel-like pools of procollagen which stained intensely with the Lillie technique.

The basement membrane of the healing gingiva of the control animals was broad and ill-defined in the early stages of healing, but became thinner and more definitive and stained less intensely as healing progressed. The basement membrane of the healing gingiva of the scorbutic animals remained broad and ill-defined, and stained a deep red-pink throughout the period of experiment. In both groups of animals the initial extension of epithelial spurs over the wound areas presented no basement membrane. The appearance of the basement membrane was associated with the maturation of the underlying connective tissue.

The elements stained by the Lillie technique are water-insoluble and alcohol-insoluble polysaccharides or polysaccharide-containing complexes in various stages of polymerization.[1] The greater intensity of staining of the procollagen and basement membrane in the healing gingiva of the vitamin C deficient animals could be interpreted as indicating a less conjugated structure of the connective tissue because more chemical groupings are available for oxidation by the metaperiodate.

## CONCLUSION

Gingival healing in experimental animals was studied with histochemical techniques. The formation and maturation of basement membrane, the appearance and increase in amount of ground substance, and the formation of collagen fibers are integral features of normal gingival healing which are impaired in acute vitamin C deficiency.

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# THE RELATIONSHIP BETWEEN DESIGN AND CUTTING EFFICIENCY OF DENTAL BURS

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## INTRODUCTION

RECENTLY several articles have been presented which have described in some degree the heat generation, the vibration characteristics, and the cutting efficiency of various rotating dental instruments.[1-6] These reports have indicated that the operating characteristics of rotating instruments are interrelated to some degree and are modified by the speed of operation and the force applied to the instrument. The previous reports have given little attention to the matter of instrument design as it is related to the cutting efficiency and functional life of the dental bur.

The cutting efficiency of a rotating dental instrument may be considered the ability of that instrument to remove a maximum amount of tooth tissue with a minimum of effort and time involved in the operation. Closely related to the cutting efficiency characteristic of the instrument is its functional life, which may be considered the time interval through which the instrument may be used effectively to cut tooth tissue. Both the efficiency and the functional life of the rotating instrument are no doubt interrelated, and both characteristics are of significance to the practitioner in his effort to shape the cavity with a minimum of time and effort.

It was the purpose of this study to determine some of the factors of design which influence the relative cutting efficiency and the functional life of the steel and carbide dental bur. Because of the manner in which the study was conducted, it has been to some degree a survey of the products available to the profession. This was not, however, the primary purpose of the investigation. It has been observed empirically by many clinical operators that there is a variation in the behavior of different products with respect to both the cutting efficiency characteristics and the relative life of the cutting instrument. It was the basic purpose of this study, therefore, to determine if there were some fundamental reasons of design which might account for this observed difference in operating behavior. Some of these differences are described in this report.

## METHOD OF STUDY

For this study, thirteen different brands of No. 557 steel and carbide burs were obtained through the local retail trade outlet. A total of nine different brands of No. 37 steel and carbide burs were obtained from the same source.

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These burs were used because previous studies on heat generation have concentrated on these two designs for comparison. Throughout this report, only code numbers are used since it is not intended to condemn or justify one product over another in this early stage of the investigation. It is of greater concern to this study to consider some of the basic factors of design which are important to favorable operating characteristics of dental burs in general.

From the standpoint of design, it is reasonable to assume that the number of teeth or flutes on the bur, the angle at which these flutes are formed, and the amount of chip clearance space ahead of the tooth are all significant to the operation of the bur. These are variables which are related to any basic design, such as the No. 557 or the No. 37 dental bur.

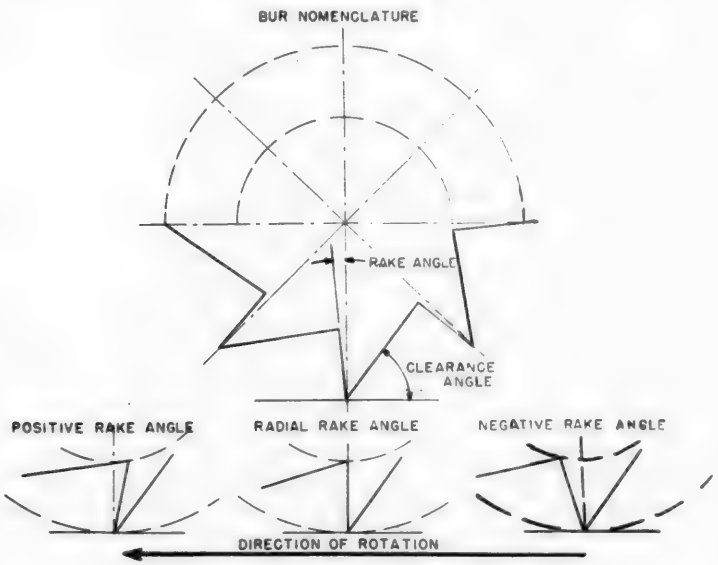
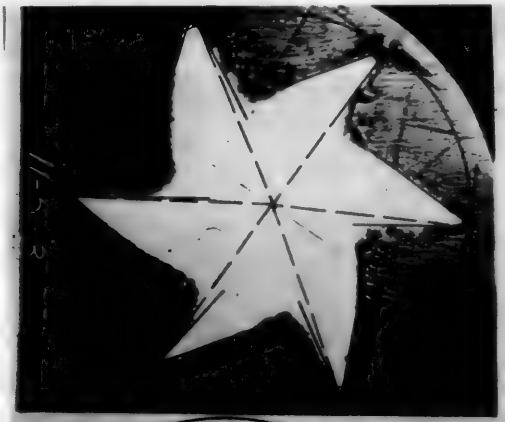



Fig. 1.—Cross-section schematic design of bur.

In Fig. 1 is shown a schematic cross-section design of a dental bur. This illustration indicates how an eight-fluted bur might be cut from round bur stock by shaping the teeth or flutes on the bur. The illustration indicates a clockwise rotation of the finished bur with various angles known as rake angles and chip clearance angles for the different teeth. When the rake angle is such that a line parallel to the face of the tooth will pass through the center of the bur, then the angle is described as a radical rake angle. As indicated in the bottom portion of the diagram, either positive or negative rake angles may be formed on the flute of the bur. The more positive the rake angle, the more effective is the cutting action of the tooth; while the more negative the rake angle, the less effective is the cutting action of the tooth. Another factor which can be observed from the central illustration in this figure is the size of the clearance angle, which is the angle on the trailing edge of the tooth. This angle may also

vary from a very shallow angle to one that is quite deep. In bur design, there is probably a proper balance between the rake angle and clearance space which gives effectiveness and life to the instrument when operated at various speeds and pressures.

Fig. 2.



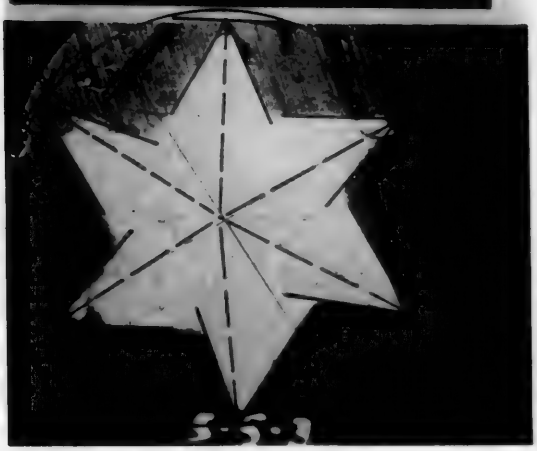



Fig. 3.

Fig. 2.—Six-tooth bur (No. 11-5-3).
Fig. 3.—Six-tooth bur (No. 5-5-3).

It has been observed that considerable variation exists among the various commercial products available with regard to both the rake angle and the chip clearance angle which are employed in the design of the burs. In order to examine the angle formed by the various teeth, four representative burs of each product were mounted in clear plastic so that the outermost 0.5 mm. end of the bur could be polished away. With the burs thus mounted, it was possible to make a cross-section examination of the basic bur design for the various products being studied.

In Fig. 2 is shown a six-tooth bur of a No. 557 design. This cross-section view shows the bur which is typical of this product to have a rake angle which approaches the radial line. Considerable chip clearance space exists in this bur, and in general it would seem well designed as an effective cutting bur. It may be questioned, however, whether or not a bur with this design will not have the tips of the teeth turned readily, or worn away, during the process of cutting hard tooth tissue.

Fig. 4.

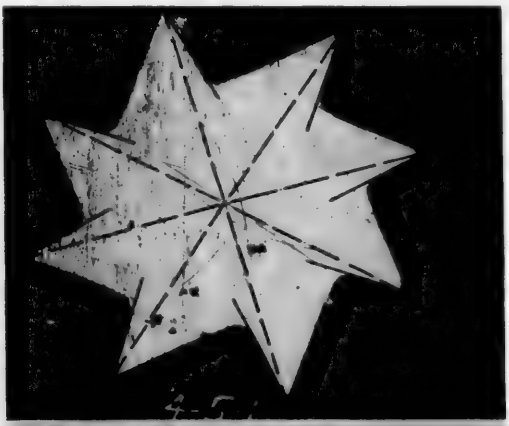


Fig. 5.

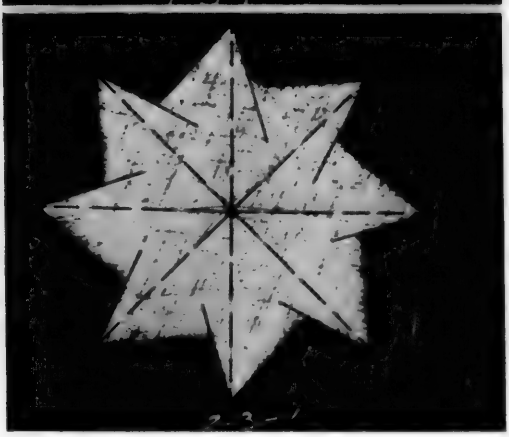


Fig. 4.—Eight-tooth bur (No. 4-5-1).
Fig. 5.—Eight-tooth bur (No. 2-3-1).

The bur represented in Fig. 3 illustrates another typical example of a six-tooth bur which is entirely different in appearance from the cross-section view of the previous bur. This bur has a pronounced negative rake angle, even though it is a No. 557 bur of essentially the same design and intended purpose as the previous bur. It would seem that this bur would offer much less positive cutting action and perhaps is less efficient in its operation, even though it may

have a longer life than the bur previously illustrated. It will be observed that this bur also has a large chip clearance angle.

It has been observed in examining various products available that six-tooth No. 557 burs often are spiral in design, whereas eight-tooth burs have relatively no spiral and often are entirely straight. An eight-tooth No. 557 bur is illustrated in Fig. 4. It can be seen that this bur has a less negative rake angle than did the six-tooth bur in Fig. 3. This variation in rake angle was observed to exist in both No. 557 and No. 37 type burs. The eight-tooth No. 37 bur, which is illustrated in Fig. 5, likewise shows an appreciable negative rake angle. The chip clearance angle in this bur also seems to be less than some illustrated in the previous figures. These cross-section views illustrate the variations which are typical of different burs that are available to the profession.

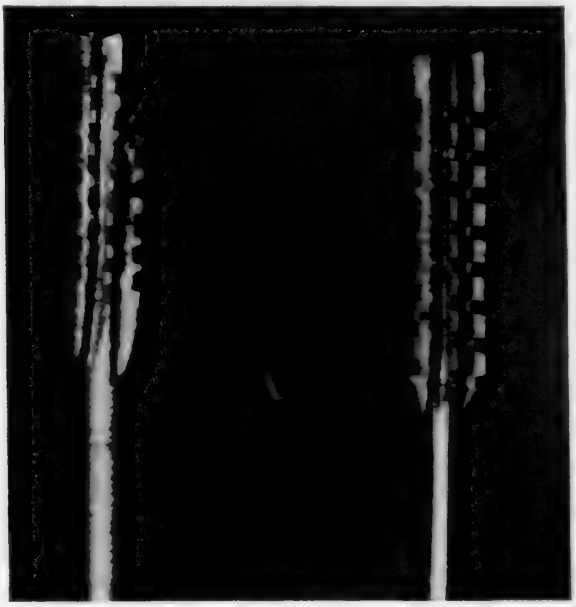


Fig. 6.—Straight fissure cross-cut burs.

Not only does the cross-section examination of various dental burs reveal that there is a difference between the various products available, but also an examination of the microscopic surface appearance of the burs varies greatly. Two eight-tooth straight No. 557 fissure cross-cut burs are illustrated in Fig. 6. The microscopic examination of the bur on the left indicates that it is less well formed and that the teeth on the bur at the right are much cleaner than those on the bur at the left. There is also a lack of uniformity in length of the head of the bur. Both burs were picked at random from their respective products and are unused.

Such variation as has been observed in the No. 557 type of bur is also common in the No. 37 type of bur. Some of the variations that exist in the

No. 37 type of bur are shown in Fig. 7. This represents three new burs which indicate variations in the cone angle, the length, and the diameter of the bur head. This represents considerable lack of standardization in products of the same basic design, and when combined with the cross-section variation in design indicated previously, it becomes evident that the operating characteristics of different products may vary greatly.

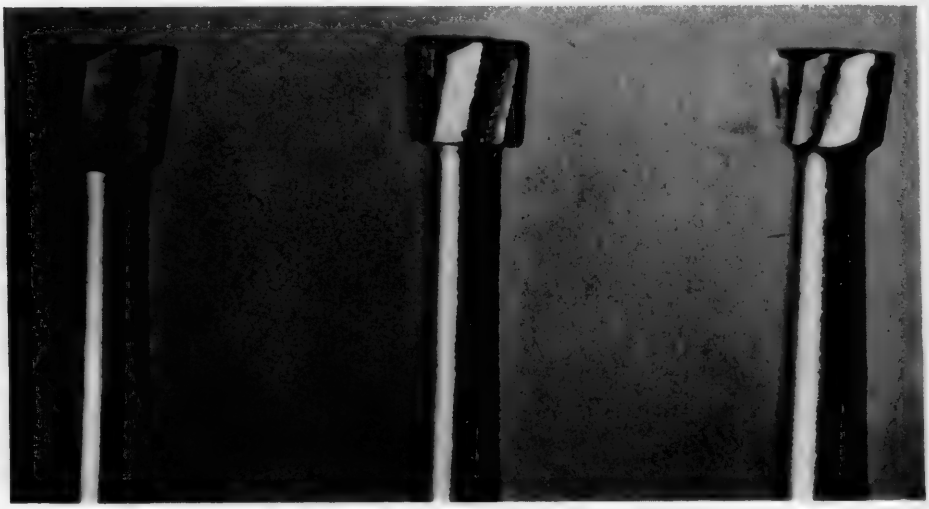


Fig. 7.—Variations in No. 37 burs.

## TOOTH ANGLES AND CHIP SPACE

Measurements of both the No. 557 and the No. 37 burs have indicated that there is a measurable variation both in the rake angle and the clearance angle of different products. In Table I are shown some of the angle values which were observed for different products. The column at the left of Table I indicates the specimen number. The top ten samples represent No. 557 steel burs, and the bottom seven represent No. 37 steel burs. The mean rake angle values are given in the second column. It will be observed that all values are negative and that the mean value varies from minus 2 to minus 16 for the No. 557 burs, and from minus 4 to minus 28 for the No. 37 burs. The range for the No. 557 burs was from a positive 6 to a minus 21 degree value, and for the No. 37 burs it was a minus 2 to a minus 30 degree value. Some extreme values are observed; for example, the No. 5-5-3 in the No. 557 series has the greatest negative rake angle, and the No. 3-3-2 in the No. 37 series has the greatest negative rake angle. The clearance angle values are shown in the column at the right. The mean for the clearance angle value in the No. 557 series was observed to be slightly greater than the mean clearance angle for the No. 37 type of bur. There is a great variation in values observed within burs of various products, as well as between burs of the different products that were examined.

TABLE I

ANGLES OF STEEL BURS

| SPECIMEN | RAKE ANGLE VALUES | | CLEARANCE ANGLE VALUES | |
|---|---|---|---|---|
| | MEAN | RANGE | MEAN | RANGE |
| No. 557: | | | | |
| 1-5-3 | −13 | − 8 to −20 | 52 | 49 to 56 |
| 2-5-2 | − 8.4 | − 8 to −10 | 56 | 55 to 57 |
| 3-5-3 | −13 | − 9 to −21 | 54 | 50 to 58 |
| 4-5-1 | −12.1 | − 9 to −14 | 54 | 49 to 59 |
| 5-5-3 | −16 | −14 to −20 | 61 | 57 to 62 |
| 6-5-1 | −11.2 | − 5 to −17 | 51 | 43 to 57 |
| 9-5-3 | −11.1 | − 7 to −15 | 57 | 51 to 62 |
| 11-5-3 | − 2 | + 6 to − 5 | 55 | 50 to 60 |
| 12-5-1 | − 5 | + 2 to −10 | 54 | 49 to 62 |
| 13-5-3 | −10.7 | − 6 to −14 | 52 | 48 to 56 |
| No. 37: | | | | |
| 1-3-1 | − 9.5 | − 6 to −18 | 48 | 41 to 56 |
| 2-3-1 | −18 | −17 to −20 | 49 | 49 to 51 |
| 3-3-2 | −28 | −25 to −30 | 45 | 43 to 50 |
| 5-3-4 | −12 | − 8 to −17 | 45 | 43 to 48 |
| 6-3-1 | − 9 | − 5 to −12 | 48 | 45 to 51 |
| 7-3-1 | − 7.4 | − 4 to −11 | 50 | 47 to 52 |
| 8-3-3 | − 4 | − 2 to − 8 | 44 | 42 to 47 |

From the photographs of the cross sections of both the No. 37 and No. 557 burs, the percentage of chip space is indicated in Table II, and was obtained by the use of a planimeter. The values in this table represent the percentage of the total area of the bur blank which has been removed during the process of formation to provide chip clearance space. For the No. 37 burs, it can be seen that the percentage of chip space varies between 31.5 per cent and 40.9 per cent for the seven products examined. In the series of No. 557 burs, the values range from 37 per cent to a maximum value of 54.4 per cent for the ten products studied. This factor of chip clearance space is important in that it provides space between the flutes for the cut particles to accumulate. The larger the chip space, the less the tendency for the clogging of the bur.

TABLE II

PERCENTAGE OF CHIP SPACE

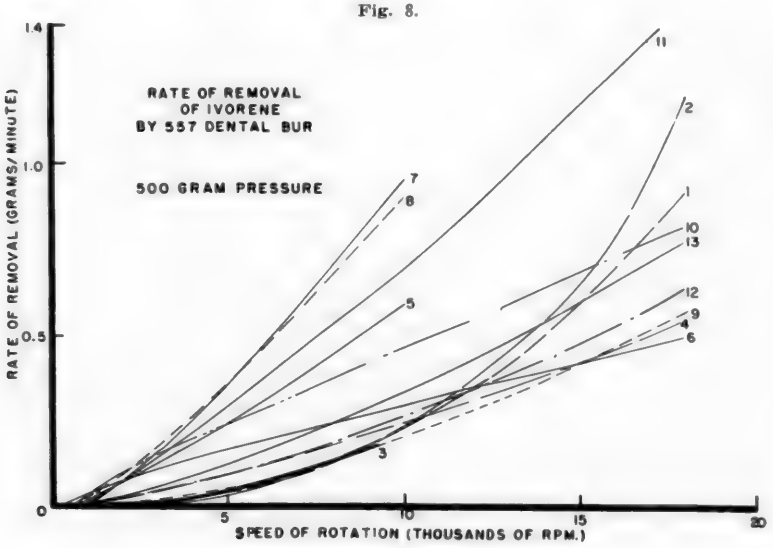
| NO. 37 BUR | | NO. 557 BUR | |
|---|---|---|---|
| BUR | PERCENTAGE OF CHIP SPACE | BUR | PERCENTAGE OF CHIP SPACE |
| 1 | 37.8 | 1 | 41.2 |
| 2 | 36.9 | 2 | 46.1 |
| 3 | 31.5 | 3 | 41. |
| 5 | 31.8 | 4 | 42.6 |
| 6 | 36.3 | 5 | 45.8 |
| 7 | 40.9 | 6 | 35. |
| 8 | 35.9 | 9 | 46.9 |
| | | 11 | 54.4 |
| | | 12 | 39.5 |
| | | 13 | 41.7 |

## RELATIVE EFFICIENCY

In order to determine the relative cutting efficiency of the various products, the different burs were used to cut the synthetic plastic material, ivorene, under

controlled pressures on the bur and with controlled speeds of rotation up to approximately 18,000 r.p.m. Relative comparisons are made in Fig. 8 which indicate the rate of removal in grams per minute when operated with a 500 Gm. load (1.1 pound) on a No. 557 type of bur at varying speeds of rotation. It will be observed from this figure that there is considerable variation in the

Fig. 8.



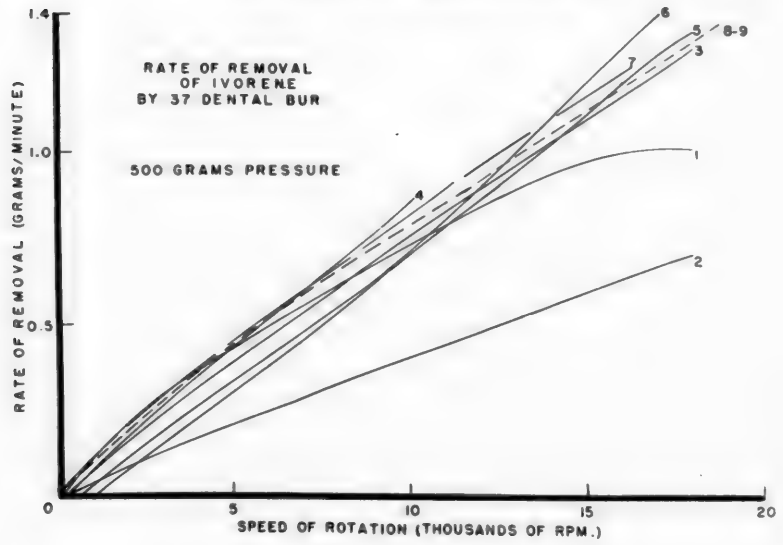




Fig. 9.

Fig. 8.—Relative effectiveness of No. 557 burs.
Fig. 9.—Relative effectiveness of No. 37 burs.

ability of different burs to cut this material. Ivorene was used for this purpose because it was relatively soft and does not tend to dull the cutting instrument, and also it does not clog the bur badly, so that reproducible values can be obtained. While it would be desirable to compare the cutting efficiency on tooth enamel or dentin, it has not been found practical to determine the weight changes which result from cutting tooth tissue. It is believed that these measurements represent relative or comparative values when cutting a homogeneous material free from the interference of dulling effect on the instrument.

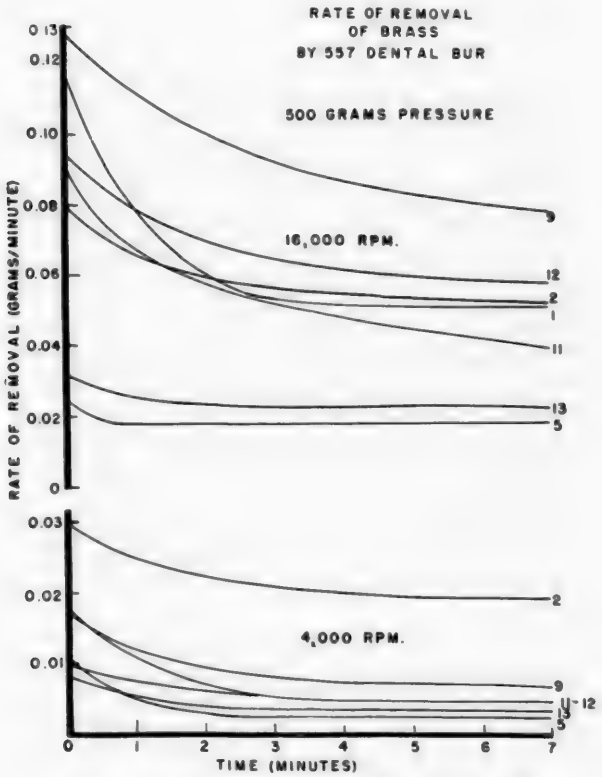



Fig. 10.—Relative life values of No. 557 steel burs.

A full significance of the curves illustrated in Fig. 8 is not yet fully known, and no effort is being made at this time to make detailed comparisons. It has been observed that the uppermost curves in this figure represent six-tooth burs. In general, they seem to be more effective in removing material than are the eight-tooth burs of the same design.

Values for the relative cutting efficiency have also been determined for the No. 37 burs. Values represented in Fig. 9 illustrate the amount of material removed with 500 Gm. load at various speeds of operation. With the No. 37

bur, there is somewhat less scatter among the various products than was observed with the No. 557 bur. In general, also, the slope of the curve is somewhat steeper, which would indicate that the No. 37 type of bur is more effective in removing material at any given speed than is the No. 557 type of bur. Likewise, no effort is made at this time to correlate the results in this figure with those in the previous tables for rake angle and chip clearance space.

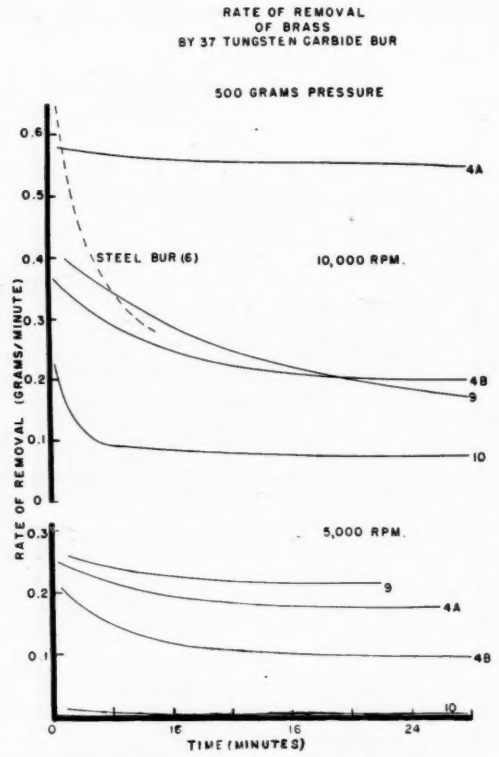



Fig. 11.—Relative life values of No. 37 carbide burs.

## COMPARATIVE LIFE VALUES

In order to determine the functional life of different burs, the relative rate of removal of brass under a given load and speed of operation was measured. In Fig. 10 are shown the relative rates of removal in grams per minute of brass, using steel burs at both 4,000 and 16,000 r.p.m. under a fixed load of 500 Gm. (1.1 pounds) on the instrument. A total of seven minutes' cutting time was used with these different burs. From this graph it can be seen that the initial cutting rate was in all instances greater than the cutting rate after one or two minutes. Some burs, like No. 5 and No. 13, were never very effective in removing material, but at the same time did not seem to dull badly during the period of operation. Other burs, like No. 1, had a high initial rate of removal,

but by the end of three minutes were only approximately one-half as effective as in the beginning.

Some studies have also been made of the relative effectiveness of carbide instruments when cutting brass. The values represented in Fig. 11 show the rate of removal of brass for a No. 37 tungsten carbide bur operated at 500 Gm. load and speeds of 5,000 and 10,000 r.p.m. for a total time of approximately twenty-eight or thirty minutes. It is seen that there is also some dulling effect on carbide burs because they do not operate as effectively after five or ten minutes as they did initially. By comparison, however, the steel burs show a much more pronounced drop in effectiveness. This is illustrated by the dotted line on this figure.

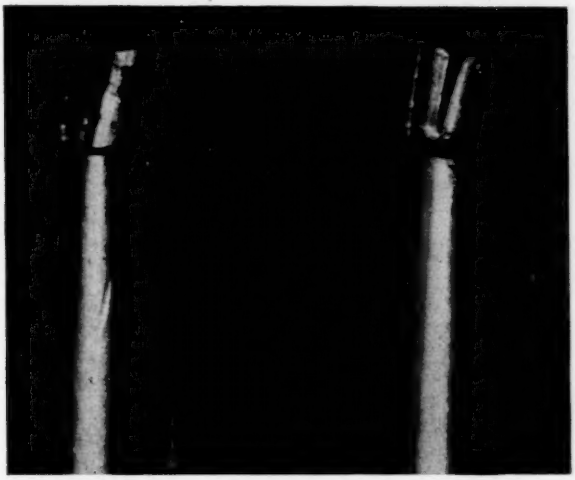


Fig. 12.—A comparison of two carbide burs.

Of some special interest is the value shown for No. 4A and 4B on Fig. 11. These two burs were of the same brand, but showed considerable difference in their effectiveness in cutting brass. A microscopic examination of these burs revealed that they were not uniform in the degree to which they had been cut during the process of formation. The illustration in Fig. 12 shows that one bur on the left was well formed, while the other on the right was only partially formed. The more completely formed bur is the most effective in removing brass during the cutting operation.

## SUMMARY

Little effort has been made at this time to correlate completely all of the basic design factors of the bur with its relative effectiveness and resistance to dulling. Some correlations appear quite evident, however, and in general, the burs with the more negative rake angle seem to be the least effective in their cutting action. At the same time, they appear to resist dulling to the greatest degree. While it is recognized that numerous other factors enter into the consideration of both

cutting effectiveness and dulling, it is believed that the basic design of the bur is of great significance and more study is being given to this problem.

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